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# XLIV. OXIDISING ENZYMES. II. THE NATURE OF THE ENZYMES ASSOCIATED WITH CERTAIN DIRECT OXIDISING SYSTEMS IN PLANTS.

By MURIEL WHELDALE ONSLOW.

*From the Biochemical Laboratory, Cambridge.*

(*Received May 6th, 1920.*)

The present and the following communications are a continuation of work undertaken upon the oxidising enzymes of various fruits for the Food Investigation Board of the Department of Scientific and Industrial Research.

In a previous paper [Onslow, 1919] it has been stated that the tissues and tissue extracts of those plants which give a blue colour with guaiacum tincture contain some substance with the catechol grouping in addition to a peroxidase. In the case of other plants the blueing of guaiacum is only brought about after addition of hydrogen peroxide, and from these plants substances with the catechol grouping are absent. It was shown, moreover, that in the first type of plants, the tissues could be freed from the "catechol" substance by thoroughly pounding and extracting with cold alcohol. A water extract of the tissue residue will then only blue guaiacum on addition of hydrogen peroxide, that is, it apparently only contains a peroxidase. Also when this extract is added to solutions of substances containing the catechol grouping, *e.g.* catechol, protocatechuic acid or the compound from the plant itself, oxidation of these takes place with a brown coloration and the production of a peroxide (probably hydrogen peroxide from decomposition in aqueous solution of a peroxide of the catechol compound). The resultant combination, peroxide and peroxidase, will then blue guaiacum. It was suggested that the enzyme present, which could only be spoken of as a peroxidase since it did not blue guaiacum without hydrogen peroxide, catalysed the oxidation of the catechol compound with the production of peroxide. Enzyme extracts prepared in a similar way from plants without catechol substances were without effect on catechol, protocatechuic acid, etc. After heating, the enzyme extract loses its power to oxidise catechol as well as the power to blue guaiacum in the presence of hydrogen peroxide. Two reactions are thus catalysed by the enzyme extract in the first of the above-mentioned classes of plants. One is the oxidation of the catechol substance by means of molecular oxygen with the formation of a peroxide: the other, the decomposition of the peroxide

with the setting free of "active" oxygen. The catalysis of the second type of reaction has always been defined as the sole function of peroxidases. If a peroxidase only is present in the enzyme extract of "catechol" plants, it must be allowed that the peroxidases in the two classes of plants are very different, in that the enzyme of one class appears to catalyse rapidly the oxidation of the catechol substance in addition to the usual function, whereas that of the other class is without effect. Since autoxidation is undoubtedly a complex process, it is not impossible that one enzyme may in some way catalyse both reactions, though the hypothesis is not satisfactory. As an alternative, it is now suggested that a second enzyme, in addition to the peroxidase, is present in the first class of plants. The function of this additional enzyme is to catalyse the oxidation of the catechol substance with the formation of a peroxide, which, when formed, is acted on by the peroxidase in the usual way. It is proposed to call this second enzyme an oxygenase[1], a term originally used by Chodat and Bach for the portion of an oxidase which can be replaced by hydrogen peroxide. Chodat [1910] speaks of oxygenases thus: "...es sind fermentartige Körper, die sich mit dem Sauerstoff der Luft zu einem Peroxyd verbinden können." Thus these authors make no clear differentiation between the organic compound from which the peroxide is formed and the enzyme which forms the peroxide, as a result, in all probability, of not having effected in their experiments a separation of the catechol substance, or similar compound, from the enzymes involved. In recent years the term oxygenase has fallen into disuse in favour of the simpler explanation of oxidase action, *i.e.* as being due to a peroxide formed by autoxidation only, and a peroxidase.

In the present communication, the following additional evidence is offered in connection with these problems.

*Spontaneous autoxidation of solutions of various phenols and aromatic acids.*

Solutions of the following substances were exposed to air for 6–7 days: adrenaline, caffeic acid (crude extract only), carvacrol, catechol, cresol (ortho- and para-), gallic acid, guaiacol, orcinol, phenol, phloroglucinol, protocatechuic acid, pyrogallol, quinol, resorcinol, tannic acid and vanillin. One per cent. solutions were used, except in the cases of adrenaline and caffeic acid, of which the strength of the solutions was unknown. In the cases of caffeic acid, gallic acid, phenol, protocatechuic acid, pyrogallol and tannic acid, before exposure to air, dilute caustic potash was added to the solutions until they just failed to change the yellow colour of bromocresol purple, *i.e.* the $P_H$ ranged between 5·2–6·8. After the long exposure to air, a few cc. were then tested for peroxide with a mixture of 5 cc. of 2 % potassium iodide, 2 cc. of 1 % acetic acid and

[1] The term oxygenase is not very satisfactory, since the function of the enzyme is not oxygen-splitting. It has been adopted for the present, however, to avoid further confusion of nomenclature. Should the enzyme be definitely isolated at some future date, the desirability of giving it a more suitable name might be considered.

1 cc. of 1 % starch solution. A blue colour appeared within half an hour in the cases of adrenaline, caffeic acid and catechol. No colour appeared in the other solutions on standing an hour. If 1 % sulphuric acid is substituted for 1 % acetic acid, in addition to the above three, the following also gave the peroxide test more or less rapidly: gallic acid, phenol, protocatechuic acid, pyrogallol, quinol and tannic acid. The remaining solutions gave no colour within the hour allowed. Controls of starch, iodide and acid also gave no colour on standing for the same length of time. It is evident, therefore, that a number of the above substances tend to form peroxides (or hydrogen peroxide by decomposition) on autoxidation, and those with the catechol grouping, on the whole, most readily. In no case did a freshly-made solution of any of the above substances give a reaction with iodide, starch and either acetic or sulphuric acid.

*Behaviour, with peroxidase, of the above autoxidised solutions of various phenols and aromatic acids as peroxides.*

The behaviour of the autoxidised solutions with a peroxidase (from horse-radish) and guaiacum was next investigated. The peroxidase solution was prepared by well pounding and extracting horse-radish root with 96 % alcohol, and then extracting the residue with water. On addition of the enzyme extract to the above solutions, followed by guaiacum tincture, a blue colour was obtained in the case of adrenaline, caffeic acid, catechol, protocatechuic acid and to some extent tannic acid. Hence such peroxides formed by autoxidation can function in the peroxide-peroxidase system, and, on the whole, those formed by compounds with the catechol grouping are most efficient. In no case was any result obtained when freshly prepared solutions were used.

*Action on freshly-prepared solutions of the above phenols and aromatic acids of the crude enzyme extracts (oxygenase and peroxidase) from plants containing a "catechol" compound.*

The potato tuber and pear fruit were used as sources of enzymes. The enzyme extracts were prepared in the usual way by rapidly pounding the tissues with cold alcohol, filtering on a pump, and repeating the process several times. A water extract of the colourless residue is used for the test. The results with extracts from both plants were as follows. A rapid browning of solutions of catechol and of caffeic and protocatechuic acids on neutralisation. On subsequent addition of guaiacum, a blue colour appeared in all three cases. A solution of adrenaline is rapidly turned red, but no blueing follows on addition of guaiacum (in contrast to the result in the last section). A solution of tyrosine is reddened (rapidly in the case of the potato, slowly with the pear) finally becoming black, but no blue colour appeared on addition of guaiacum at any stage. A solution of *p*-cresol is turned orange (rapidly with potato, slowly with pear), but unlike the case of tyrosine, a blue colour is

given on subsequent addition of guaiacum. No action was obtained with the remainder of the substances.

The reactions with tyrosine and *p*-cresol are presumably due to tyrosinase which has long been known to be present in the potato, though no previous reference can be discovered to its occurrence in the pear fruit. The reaction with adrenaline may be due, either to the presence of tyrosinase, which, according to some authors, acts on this substance, or to the presence of the oxidising enzyme which reacts with the catechol grouping, or to both. Points of interest are the non-blueing of guaiacum in the case of adrenaline and tyrosine, whereas it occurs in the case of *p*-cresol. Also the fact that when an autoxidised solution of adrenaline is used with a peroxidase, as in the last section, blueing occurs on subsequent addition of guaiacum.

*Separation of two enzymes, oxygenase and peroxidase, from the enzyme extract of the potato.*

In view of the suggestion that two enzymes, an oxygenase and a peroxidase are present in the pear and potato, an attempt was made to separate them by fractional precipitation. Such a method was originally employed by Chodat and Bach [1903: Bach, 1910] for separating their oxygenase component from the peroxidase in the case of an extract from the fungus, *Lactarius* (Basidiomycetes). Their extract was precipitated with 40 % alcohol which produced a precipitate rich in oxygenase, but poor in peroxidase. After continued fractional precipitation with alcohol, a final precipitate was obtained containing peroxidase only.

The method adopted for the potato was as follows. The tuber was pounded and extracted with cold alcohol as previously described. The residue was then extracted for an hour with about three times its weight of water, and the extract squeezed out through muslin. The extract was then centrifuged, the opalescent solution decanted and absolute alcohol added until the concentration reached 40 %. The precipitate taken up with water gave in several experiments a stronger oxygenase reaction (blueing with catechol solution and guaiacum in the presence of peroxidase) than peroxidase reaction (blueing with hydrogen peroxide and guaiacum). Reprecipitation of the first fraction failed to remove the peroxidase, nor was the result more successful by making the concentration of alcohol 20 or 30 % for the first precipitation. By further precipitation of the first filtrate four or five times with additional volumes of alcohol equal to that first added, a precipitate was eventually obtained which ceased to give the oxygenase reaction though it still gave a good peroxidase reaction. By adding saturated ammonium sulphate (20 cc. to 80 cc. of extract) instead of alcohol for the first precipitation, in one trial a first fraction was obtained which gave only a slight peroxidase, but a strong oxygenase reaction. The experiment, however, could not be repeated with any certainty.

It therefore appears that the method of fractional precipitation is not wholly satisfactory for the separation of the two enzymes. More recent experiments of Bach [1916] would seem to confirm this result since he has tried to separate Chodat and Bach's two components of *Lactarius* oxidase by ultrafiltration. Of the two, he found the peroxidase to be much the more rapidly adsorbed by ultrafiltration. The oxygenase remains on the ultrafilter, and its oxidising power can be regenerated by addition of horse-radish peroxidase.

The above results furnish evidence in favour of the existence of two enzymes in plants which contain a catechol substance, turn brown on injury and blue guaiacum without addition of hydrogen peroxide. In plant tissues, in cases so far investigated, we conclude then that three factors are necessary for the blueing of guaiacum: an aromatic substance with a dihydroxy-grouping in the ortho-position; an enzyme, oxygenase, which catalyses the oxidation of such substances with the production of an organic peroxide (or hydrogen peroxide by decomposition); and, finally, a peroxidase which transfers "active" oxygen to readily oxidisable compounds, *e.g.* guaiacum. Artificially, the place of the oxygenase and aromatic compound can be taken either by hydrogen peroxide or by peroxides formed by the autoxidation of certain substances. If the oxidase (synonymous with laccase, phenoloxidase and phenolase) of the pear and potato is to be defined as an enzyme blueing guaiacum, then all three components are essential to its constitution, for the mixture of enzymes alone, oxygenase and peroxidase, is unable to blue guaiacum unless contaminated with the catechol substance.

It is probable that the oxidases (laccases, phenoloxidases, phenolases) obtained by previous workers by precipitation of plant juices with alcohol, are in many cases mixtures of oxygenase and peroxidase with adsorbed catechol compound. Such mixtures would blue guaiacum, and would transfer oxygen to many other substances, such as phenols and aromatic acids, to a greater or less degree. The enzyme mixture, oxygenase and peroxidase (which needs care to be prepared free from catechol compound), has, by virtue of the oxygenase, so far been found only to act on the dihydroxy-grouping in the ortho-position. It can be readily seen that the combination, catechol compound and oxygenase, behaves in every way like Chodat and Bach's organic substance of an enzyme-like nature endowed with the power of forming peroxides, which they call oxygenase.

## Conclusions.

1. Solutions of catechol, caffeic acid, protocatechuic acid and adrenaline, compounds which have the dihydroxy-grouping in the ortho-position, tend to autoxidise slowly when left in air with the formation of peroxides.

2. When a solution of a peroxidase is added to any of the above peroxides, the combination constitutes an oxidase system which will blue guaiacum.

3. The autoxidation, with production of peroxides, of catechol, protocatechuic acid and caffeic acid is accelerated by enzyme extracts of plants which turn brown on injury, and of which the juices blue guaiacum without addition of hydrogen peroxide.

4. Such plants have been found to contain a compound giving the "catechol" reaction. It is suggested that they also contain, in addition to a peroxidase, a second enzyme, which will be termed an oxygenase. The function of the latter is to catalyse the autoxidation of the "catechol" compound with the formation of a peroxide.

5. Three components therefore, in the cases investigated, are present in what has been termed an oxidase (laccase, phenoloxidase or phenolase): a "catechol" compound from which a peroxide can be formed, and two enzymes, an oxygenase which catalyses the production of peroxide, and a peroxidase which decomposes the peroxide with the formation of "active" oxygen. All three components are essential for the blueing of guaiacum.

6. The peroxidase can be separated from the oxygenase by fractional precipitation with alcohol, though the converse has not been accomplished.

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# XLV. OXIDISING ENZYMES. III. THE OXIDISING ENZYMES OF SOME COMMON FRUITS.

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(*Received May 6th, 1920.*)

In carrying out a general survey of the oxidising enzymes of fruits, certain additional observations have been recorded.

The first point to be emphasised is the fact that divergent results may be obtained in testing for oxidising enzymes, according to whether tests are made on the fresh tissue, or on extracts prepared in various ways from the tissues. Some workers have obtained their results by placing the tissues in the testing reagents. Others have used tissue extracts only. It appears desirable to examine the materials in both ways.

The maximum "oxidising capacity" of the tissue is usually shown when the tissue is placed directly in the reagent. In the case of extracts, the reaction may be modified by the following conditions. First, the enzyme itself may not be extracted; secondly, the relative concentration of enzyme, sugars, organic acids and tannins occurring in the extracts are different from those in the cells, and the "oxidising capacity" of the tissue is not always shown until special steps have been taken to remove some of the products present in solution. In order to understand, however, the nature of the reactions *in situ*, extraction of the various components of the system, its inhibitors, etc., is essential.

Further, it may happen that certain substances, such as organic acids, tannins, etc., prevent even the tissues from giving the reactions, and these must be removed by solvents, *e.g.* alcohol, leaving the enzymes in the tissue residue. Moreover, sometimes the enzymes may be adsorbed by the tissue residue and not readily extracted. In this case, the reactions will only occur when the fragments of the residue are placed in the reagents, the enzyme and reacting substances having been adsorbed on to these surfaces.

It has been stated by various observers that tannins have an inhibiting effect on oxidising enzymes, and the present results confirm this observation. It is further shown that malic acid to the extent in which it occurs in many fruits, even in the form of neutral salts, may also have an inhibiting effect.

Observations have been made on the fruits according to the following procedures. (*a*) *Reactions of tissues.* Pieces of tissue are placed in the reagents,

*viz.* alcoholic solution of guaiacum (with or without hydrogen peroxide): 1 % solution of benzidine in 50 % alcohol with hydrogen peroxide; 1 % solution of $\alpha$-naphthol in 50 % alcohol with hydrogen peroxide. (*b*) *Reactions of water extract.* A water extract of the pounded tissues is tested with the above reagents. If acid, it is neutralised to litmus. (*c*) *Reactions with extract of enzymes.* The tissues are pounded rapidly with cold 96 % alcohol, sucked dry on a filter funnel, and the process repeated several times. The residue ("tissue residue") is extracted with water, and in this way a crude extract ("enzyme extract") of the enzymes practically free from sugars, tannins and organic acids is obtained. The enzyme extract is tested for peroxidase with guaiacum and hydrogen peroxide, and for oxygenase with catechol and guaiacum[1]. (*d*) *Extraction of aromatic compounds and their reaction with the enzyme extract.* The tissues are extracted with hot alcohol and the alcohol removed by distilling *in vacuo*. The residual extract is diluted with a little water and precipitated with powdered lead acetate. The precipitate so formed chiefly consists of salts of aromatic substances (including tannins) and organic acids. It is decomposed with the minimal amount of sulphuric acid and filtered from lead sulphate. The filtrate is neutralised to litmus, and tested for the catechol reaction with a few drops of ferric chloride followed by a few drops of a 1 % solution of sodium carbonate. A green colour becoming blue, purple or red on addition of the carbonate denotes the presence of a substance with the catechol grouping. To another portion some enzyme extract [see (*c*) above] is then added. If a catechol reaction has been given and an oxygenase has been detected, a brown colour may be produced in the mixture followed by a blue colour on adding guaiacum; this denotes that the whole oxidase system is present. In a few cases such a result is only obtained by using the tissue residue itself. If both oxygenase and catechol substance have been detected and yet no oxidase system can be synthesised in the above way, the absence of the reaction is usually due to the presence of organic acids or tannins.

If considerable quantities of malic acid are present, this is removed by adding calcium chloride solution, and then excess of alcohol, to the neutralised filtrate obtained by treating the lead salts with dilute sulphuric acid. The calcium malate is insoluble in alcohol and can be filtered off. The alcohol is removed by distilling *in vacuo*, and the residue tested with the enzyme extract.

If tannin is present, this is removed by adding a little concentrated gelatin solution. After filtering, the filtrate is tested with the enzyme extract.

Employing the above methods, the following fruits have been investigated. The results may vary in detail, according to the variety or ripeness of the fruit used.

I. The Apple (*Pyrus malus*). The fruit turns brown on injury. (*a*) *Reactions of tissues.* In guaiacum tincture the tissue usually turns blue, though in some varieties the reaction may be slow. It is accelerated by bruising the tissue.

[1] Since peroxidase has always been present with oxygenase, it has not been necessary to supply a peroxidase from another source.

On addition of hydrogen peroxide, the reaction is obtained at once. The benzidine and α-naphthol tests are positive. (*b*) *Reactions of water extract.* All the tests are positive, though the reaction with guaiacum alone may be delayed or slight. (*c*) *Enzyme extract.* This gives both the peroxidase and oxygenase reactions. (*d*) *Extraction of aromatic compounds and their reaction with enzyme extract.* When the solution, obtained by decomposition with sulphuric acid of the lead salts of the aromatic compounds and malic acid, is neutralised, a yellowish precipitate is obtained. On taking up this precipitate in very dilute sulphuric acid, the solution gives a strong catechol reaction and is precipitated by gelatin; the solution also does not react with the enzyme extract. The substance precipitated appears to be a catechol tannin which is soluble in alcohol and the dilute acid of the fruit itself, but is insoluble in water. The filtrate from the tannin precipitate also gives the catechol reaction, though less strongly. When tested with enzyme extract and guaiacum no blueing is obtained. The filtrate is then treated with 2–3 cc. of a concentrated calcium chloride solution and excess of alcohol added. A white flocculent precipitate of calcium malate is formed. This is filtered off and the alcohol removed from the filtrate by distilling *in vacuo*. The residue taken up in water still gives a catechol reaction. With tissue residue and guaiacum it gives a blue colour. With the enzyme extract and guaiacum it may give a positive reaction if the liquid is slightly acid to litmus.

*Conclusion.* The apple fruit contains an oxidase, that is an oxygenase, a peroxidase, and an aromatic substance with the catechol grouping. The oxygenase will activate the oxidation of the catechol aromatic compounds *in situ* in the cell with the production of a brown colour and of a system which will blue guaiacum: it will also activate the oxidation of catechol supplied artificially with the production, in the presence of the peroxidase, of a similar system. A large part of the aromatic compounds of the fruit appears to be in the form of a catechol tannin, and this cannot be activated by the enzyme *in vitro*. After removing the tannin and malic acid, the residuum of aromatic compounds left is activated by the oxygenase, with difficulty in solution, but more readily when fragments of the tissue residue are suspended in the solution.

II. The Quince (*Pyrus cydonia*). The cut surface of the fruit turns brown less readily than that of the apple, but on pounding in a mortar, considerable browning is produced. (*a*) *Reactions of tissues.* The tissue gives no blue colour with guaiacum tincture, except on adding hydrogen peroxide. The benzidine and α-naphthol tests are positive. (*b*) *Reactions of water extract.* This gives none of the above tests even after neutralisation. (*c*) *Enzyme extract.* This gives the peroxidase but only a slight oxygenase reaction, unless fragments of tissue residue are added, when they become deep blue. (*d*) *Extraction of aromatic compounds and their reaction with enzyme extract.* The procedure and reactions are very similar to those for the apple. In the same way, though in greater quantity, the fruit appears to contain a catechol tannin which is

soluble in alcohol and dilute acids. On neutralising the filtrate (from the decomposition of the lead salts) this tannin is precipitated. The filtrate from the tannin precipitate still gives the catechol reaction but contains no perceptible amount of tannin. It, however, gives no blue colour with guaiacum and either the enzyme extract or tissue residue. After removing the malic acid as calcium malate and distilling the filtrate *in vacuo*, the residue gave the catechol reaction less strongly, but would again give no blue colour with guaiacum and either the enzyme extract or tissue residue.

*Conclusion.* The quince fruit contains an oxidase (oxygenase, peroxidase and aromatic substance with the catechol grouping). The oxygenase will activate the oxidation of the catechol aromatic compounds *in situ* in the cell causing browning, but the blueing of guaiacum is largely inhibited owing to the tannin and malic acid present. The oxygenase is not readily extracted from the tissue residue, so that only this residue itself gives a good reaction with catechol solution and guaiacum. No reactions could be obtained between the plant aromatic compounds and enzymes *in vitro* even when the tissue residue was employed. A neutralised water extract of the tissues gives no reactions, probably because of the inhibitory action of the malates.

III. The Pear (*Pyrus communis*). The fruit turns brown readily on injury. (*a*) *Reactions of tissues.* The tissue turns blue with guaiacum tincture and gives positive reactions with the benzidine and $\alpha$-naphthol tests. (*b*) *Reactions of water extract.* Positive reactions with all three tests. (*c*) *Enzyme extract.* This gives both the peroxidase and oxygenase reactions. (*d*) *Extraction of aromatic compounds and their reaction with enzyme extract.* The filtrate, after decomposing the lead salts with sulphuric acid, gives no precipitate on neutralisation, and a very slight, if any, reaction for tannin. It gives a strong catechol reaction. On addition of the enzyme extract, browning occurs and a deep blue colour on subsequent addition of guaiacum.

*Conclusion.* The pear fruit contains an oxidase (peroxidase, oxygenase and aromatic substance with the catechol grouping). The oxygenase will activate the oxidation of catechol artificially supplied, with the production, in the presence of the peroxidase, of the oxidase system. When the separate extracts of aromatics and enzymes are reunited, the oxidase system is obtained.

A comparison of the oxidase reactions of the three fruits is rendered more comprehensible if the average percentages [Wehmer, 1911] of tannin and free acids are noted.

| | Pear | Apple | Quince |
|---|---|---|---|
| Organic acid (chiefly malic) ... | 0·1–0·2 % | 0·7–1·2 % | 0·84–3·5 % |
| Tannin ... ... ... ... | 0·05 | 0·1–0·3 | |

(No reference to the percentage of tannin in the quince could be found, but large quantities were obtained on extraction.) The tannin and organic acids inhibit the oxidising reactions in both the tissues and extracts, though more so in the latter, the effect of the tannin being to precipitate the enzymes. The presence of tannin also renders the extraction of the enzymes more difficult,

even after the bulk of the tannin is removed by alcohol. This is especially so in the case of the oxygenase, *e.g.* in the quince, which appears to be precipitated and adsorbed by the tissue fragments.

IV. The Plum (*Prunus domestica*). Both red and purple varieties were investigated separately. The fruit turns brown on injury. (*a*) *Reactions of tissues.* The tissues give a positive reaction with guaiacum alone, and also with both benzidine and α-naphthol tests. (*b*) *Reactions of water extract.* The same results as in (*a*) were obtained. (*c*) *Enzyme extract.* This gives both peroxidase and oxygenase reactions. (*d*) *Extraction of aromatic compounds and their reaction with enzyme extract.* A heavy whitish precipitate (red plums) or purple precipitate (purple plums) is obtained with lead acetate. After decomposition with sulphuric acid and neutralisation, the extract gives a good catechol reaction, but no reaction with the enzyme extract and guaiacum. After removal of the malic acid, the extract still gives the catechol reaction and, on addition of the enzyme extract and guaiacum, a blue colour. The latter reaction is better if the mixture is slightly acid to litmus.

The same results were obtained with greengage and damson fruits.

The percentages of acid and tannin are as follows: acid, chiefly malic (plum, 0·77–1·01 %; greengage, 0·82 %; damson, 0·92 %); tannin (damson, 0·04 %) [Wehmer, 1911].

*Conclusion.* The plum, greengage and damson all contain an oxidase (peroxidase, oxygenase and aromatic substance with the catechol grouping). Hence the tissues brown on injury and give the oxidase reaction. The oxygenase will activate the oxidation of catechol solution with the production of a peroxide, etc. The extracted aromatic compounds and enzymes from the fruit when recombined will give a blue reaction with guaiacum after the removal of the malic acid.

V. The Banana (*Musa sapientum*). On injury, *e.g.* pounding in a mortar, the skin turns brown but the flesh is less affected. The results recorded below given by the flesh of the fruit varied somewhat in individual fruits and in fruits of different degrees of ripeness. On the whole, however, the reactions were as stated. (*a*) *Reactions of tissues.* The skin turns blue with guaiacum alone: the flesh only after addition of hydrogen peroxide. Both skin and flesh give positive reactions with the benzidine and α-naphthol tests. (*b*) *Reactions of water extract.* The extract of the skin gives a positive reaction with guaiacum alone: that of the flesh no reaction until hydrogen peroxide is added. Extracts of both skin and flesh give positive reactions with the benzidine and α-naphthol tests. (*c*) *Enzyme extract.* From both skin and flesh these give the peroxidase and oxygenase reactions. (*d*) *Extraction of aromatic compounds and their reaction with enzyme extract.* The solution derived from decomposition with sulphuric acid of the lead salt from the skin gives the catechol reaction. On addition of the enzyme extract from the skin followed by guaiacum, a blue colour is obtained. The reaction takes place more readily after precipitation with gelatin owing to the presence of a certain amount of

tannin. The solution from the decomposition of the lead salt from the flesh gives a doubtful catechol reaction, and no blue colour with the enzyme extract from the flesh followed by guaiacum. After removal of malic acid by the usual method, the residue gives no catechol reaction and again no result after addition of enzyme extract and guaiacum.

*Conclusion.* (The results, as mentioned above, given by extracts of enzymes and aromatic compounds of the flesh vary, but on the whole they may be represented as follows.) Both the skin and flesh of the fruit contain peroxidase and oxygenase. The skin also contains substances with the catechol grouping, but these are practically absent from the flesh. The oxygenase of both skin and flesh catalyses the oxidation of catechol, artificially supplied, with great rapidity followed by blueing on addition of guaiacum. The skin oxygenase also activates the oxidation of its own aromatic compound with the production of a blue colour on addition of guaiacum. No reaction could be obtained between the enzyme and aromatic extracts of the flesh.

VI. The Orange (*Citrus aurantium*). The fruit does not brown on injury. (*a*) *Reactions of tissues.* Rind: positive result with guaiacum and hydrogen peroxide and with the other two tests. Pulp: slight local positive reaction with benzidine and $\alpha$-naphthol tests only. (*b*) *Reactions of water extract.* Rind: positive reaction only with benzidine test. Pulp: after neutralisation to litmus, no reactions with any of the three tests. (*c*) *Enzyme extract.* Both rind and pulp give some positive reaction with guaiacum and hydrogen peroxide and with the benzidine test. Neither gives the oxygenase reaction. (*d*) *Extraction of aromatic compounds.* No catechol reaction could be detected.

VII. The Lemon (*Citrus limonum*). The fruit does not brown on injury. (*a*) *Reactions of tissues.* Rind: positive reaction with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction with benzidine and $\alpha$-naphthol tests only. (*b*) *Reactions of water extract.* Rind: positive reaction with benzidine and $\alpha$-naphthol tests only. Pulp: after neutralisation to litmus, positive reaction with benzidine test only. (*c*) *Enzyme extract.* Rind: good positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction only with benzidine test. With the other two tests positive reaction only if tissue residue is used. Neither extract gives the oxygenase reaction. (*d*) *Extraction of aromatic compounds.* No catechol reaction could be detected.

VIII. The Lime (*Citrus limonum*, var. *acida*). The fruit does not brown on injury. (*a*) *Reactions of tissues.* Rind: positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: positive reaction with the benzidine test only. (*b*) *Reactions of water extract.* Not tried. (*c*) *Enzyme extract.* Rind: positive reactions with guaiacum and hydrogen peroxide and with the other two tests. Pulp: negative reactions with all three tests but positive reaction with the benzidine test if the tissue residue is used. Neither extract gives the oxygenase reaction. (*d*) *Extraction of aromatic compounds.* No catechol reaction could be detected.

*Conclusion.* The fruits of the orange, lemon and lime contain a peroxidase in both the rind and the pulp. No substance giving the catechol reaction could be detected in any of the fruits and there is no oxygenase. In the rind the peroxidase reaction is strongest in the lime, less in the lemon and least in the orange. From the pulp, the peroxidase is most readily extracted in the case of the orange, less in the lemon and least in the lime. These fruits afford an example of the possibility of obtaining a greater oxidation reaction with the crudely separated peroxidase than with the tissue itself.

IX. The Raspberry (*Rubus idaeus*). The fruit does not brown on injury. (*a*) *Reactions of tissues.* Some positive reaction only with benzidine and *a*-naphthol tests. (*b*) *Reactions of water extract.* These cannot be detected because of the anthocyan pigment. (*c*) *Enzyme extract.* Positive reactions with guaiacum and hydrogen peroxide and with the other two tests. No oxygenase reaction is given. (*d*) *Extraction of aromatic compounds.* No catechol reaction could be detected.

*Conclusion.* The raspberry fruit contains a peroxidase but no oxygenase and no substance giving the catechol reaction. This fruit affords another example of greater oxidation powers of crudely prepared peroxidase than of the tissue itself.

My thanks are due to Dr F. F. Blackman, F.R.S., for kind criticism and advice in connection with this and the preceding paper.

REFERENCE.

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# XLVI. A STUDY OF SOME BIOCHEMICAL TESTS. No. 2. THE ADAMKIEWICZ PROTEIN REACTION. THE MECHANISM OF THE HOPKINS-COLE TEST FOR TRYPTOPHAN. A NEW COLOUR TEST FOR GLYOXYLIC ACID.

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In 1874 Adamkiewicz published [1874] a description of the colour phenomena due to the action of strong sulphuric acid on egg-albumin. In some experiments he used acetic acid as a solvent, and he observed in many such cases that a purple colour was produced on the addition of the sulphuric acid. He considered this colour to be due to "a special influence of the acetic acid upon the colour already produced in the sulphuric acid protein solution."

Udranszky [1888] described the test as a furfural reaction, which explanation was generally accepted till Hopkins and Cole [1901, 1, 2] published the first of their well-known papers, in which they showed that the colour was due to an impurity in the acetic acid. They isolated glyoxylic acid from several samples of acetic acid and showed that in the presence of sulphuric acid it gave a purple colour with certain proteins, the facts thus discovered leading later to the isolation of the amino-acid, tryptophan, which is responsible for the reaction.

Cole [1903] investigated the various protein colour tests with aldehydes, or bodies that gave rise to aldehydes, and proved that the majority of these reactions depended on the presence of tryptophan. He drew attention to the importance of the presence of an oxidising agent in the production of the colours.

Hopkins and Cole's explanation was criticised by Rosenheim [1906], who described a colour reaction between tryptophan and formaldehyde in sulphuric acid containing a trace of an oxidiser, which was similar to that given by glyoxylic acid. Rosenheim considered that the Hopkins-Cole test depended on the presence of formaldehyde in the acetic acid or arising from the reagents used. He remarked upon the ease with which glyoxylic acid is decomposed at high temperature into formaldehyde and carbon dioxide.

Dakin [1907] investigated the tests, and found that glyoxylic acid prepared by different methods gave the test. He thought it "hard to believe that these uniformly positive results with different specimens of glyoxylic acid are due to impurities." Glyoxylic acid preparations in many cases gave the test directly, formaldehyde always required an oxidising agent. Dakin discussed the possibilities of the production of glyoxylic acid from formaldehyde by an aldol type of condensation followed by oxidation. Kondo (1906) described a test for indole similar in many respects to the Rosenheim test. Formaldehyde with indole gives a crimson colour in presence of strong sulphuric acid.

Research was done in the following years on the general chemistry of bodies related to tryptophan, which will be referred to later, as it does not directly concern the Hopkins-Cole reaction [Blumenthal, 1909].

In 1913 Homer published the results of work on aldehyde derivatives of tryptophan [1913]. She believed that the colour bodies were due to an interaction between the aldehyde and the imino-group of the tryptophan. She concluded from the general behaviour of these bodies that the Hopkins-Cole test was due to formaldehyde.

In the same year Mottram [1913] described a failure of the glyoxylic test owing to an excess of oxidising agents in the sulphuric acid used. This was confirmed by Breidahl in 1915, who stated that such acids could be made suitable for the test by the addition of small quantities of reducing agents [1915]. These papers emphasised the fact that a trace of an oxidising agent is necessary in the majority of cases; if the oxidising agent be too strong or too weak the test will not succeed.

Voisenet in 1918, from similarities in appearance and behaviour towards reagents, concluded that the colour produced in the Hopkins-Cole and the Rosenheim tests was the same substance, and that in each test it was due to formaldehyde [1918].

The following research was undertaken originally with the object of finding a satisfactory method for estimating tryptophan colorimetrically. Although such a method has not yet been found, some information has been obtained about the mechanism of the tryptophan colour tests. The first question investigated was the nature of the aldehydes concerned in the Hopkins-Cole and Rosenheim tests with a view to deciding the long-standing glyoxylic acid and formaldehyde controversy.

*The Hopkins-Cole Reaction in the absence of Formaldehyde.* If formaldehyde were liberated from the glyoxylic acid during the process of the test it should be possible to detect its presence, since there are colour tests for formaldehyde in presence of sulphuric acid that are more delicate than the reaction between formaldehyde and tryptophan. Should formaldehyde be detected under the conditions of the Hopkins-Cole reaction, it probably enters into the test. On the other hand, should the test be obtained under conditions that are such that formaldehyde cannot be detected in the reacting solutions, it is strong evidence that the reaction is due to glyoxylic acid.

Glyoxylic acid is more stable in strong sulphuric acid than has been generally supposed. When calcium glyoxylate was added to concentrated sulphuric acid and the mixture warmed gently, decomposition did not begin until the temperature rose above 50°. Carbon dioxide was then evolved, and formaldehyde was recognised in the solution by means of the thiophen test [Fearon, 1918]. The Hopkins-Cole test may be carried out at temperatures below that of the decomposition of the glyoxylate and in the absence of water, by using solid tryptophan and calcium glyoxylate. The characteristic colour develops under conditions where formaldehyde can be shown to be absent. (In passing, it may be noted that if the sulphuric acid be contaminated with nitrous acid an ivy-green colour may be formed on warming the solution, this is due to an entirely different reaction which will be discussed in a later communication.)

Various samples of acetic acid which gave the reaction were tested for formaldehyde, alone and under the conditions of the test, with negative results in nearly every case.

To detect the presence of formaldehyde in excess of sulphuric acid the following tests were employed.

*The Thiophen Test* (1918). This test will show one part of formaldehyde in half a million parts of sulphuric acid. The colour with formaldehyde is distinctive and much more delicate than the colour with glyoxylic acid.

The delicacy of Rosenheim's test for tryptophan is about 1 in 200,000 at most.

*The Pyrogallol-aldehyde Reaction*, described in the present paper.

This is an extremely sensitive test for aldehydes in presence of sulphuric acid; it will show 1 part formaldehyde in over 1,000,000 parts water, and may be used to show the presence of formaldehyde in mixtures containing glyoxylic acid.

As the result of a series of experiments on the Hopkins-Cole test under various conditions and with different preparations of glyoxylic acid, it is concluded that the test can be obtained in the absence of formaldehyde.

*The Rosenheim Test in the absence of Glyoxylic Acid.* Although glyoxylic acid gives many of the general aldehyde reactions, there is no simple colour test for distinguishing it from other aldehydes in presence of sulphuric acid. The test current in the text-books is simply the reverse of the Hopkins-Cole reaction and open to the same criticism of uncertainty as regards the aldehyde. This test was introduced by Eppinger [1905] and modified by Schloss [1906].

## A New Colour Test for Glyoxylic Acid.

The reagent is 1 % pyrogallol in pure concentrated sulphuric acid, which must be free from any nitrous contamination, otherwise the solution turns brown and is useless. The reagent will not keep for long, after a few days a white condensation product is deposited.

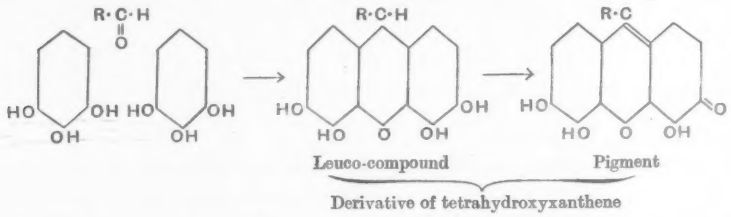
When a trace of calcium glyoxylate, or a couple of drops of reduced oxalic acid solution are added to the reagent, a deep blue colour develops on gentle warming. Dilution with water changes the blue to a deep carmine which becomes blue again on addition of excess of sulphuric acid.

The blue colour is not given by oxalic acid or any of its simple derivatives which do not give rise to glyoxylic acid under the condition of the test.

With the aliphatic aldehydes the reagent gives various shades of red. The presence of glyoxylic acid along with formaldehyde can be shown by the production of a purple due to the formation of two colours. The test is useful for detecting aldehydes in ether, as mentioned later. Syrupy phosphoric acid may be used instead of sulphuric acid as a condensing agent, in which case a red colour is obtained with all the aldehydes on warming.

The delicacy of the test is greatest for formaldehyde; with glyoxylic acid it will show less than one part in a thousand, with weaker solutions the colour takes some time to develop.

The chemistry of the test has only been briefly investigated as yet. The coloured condensation compounds are probably derivatives of xanthene. When separated from the sulphuric acid they show many of the properties of xanthydrol, such as forming typical insoluble compounds with urea in acid solution. The colour is probably due to the formation of the pyrone ring [Palazzo and Onorato, 1905, and references].




On applying the pyrogallol-reagent to solutions of formaldehyde in sulphuric acid in the presence of oxidising agents, under the conditions of the Rosenheim test, no evidence whatever could be obtained of the formation of glyoxylic acid, though on addition to the mixture it could be detected. This supports Rosenheim's view that the test depends on formaldehyde alone.

From a purely chemical aspect, it is not improbable that both formaldehyde and glyoxylic acid should react with tryptophan to give distinct derivatives, though subsequent investigation showed that the glyoxylic derivative readily lost carbon dioxide and changed into the corresponding formaldehyde derivative as might be expected. The fact that the Hopkins-Cole test is considered by many investigators to be more reliable than the Rosenheim test would be explained by the greater tendency, as subsequently observed, of the formaldehyde to react still further with the condensation products. A disadvantage of both tests is the local heating at the junction of the liquids;

this can be greatly reduced by employing glacial acetic acid as a solvent for the glyoxylate or the formaldehyde as the case may be. As will be shown later, an oxidising agent is necessary in both tests, this is usually supplied by the sulphuric acid [Mottram, 1913].

### The Mechanism of the Hopkins-Cole Reaction.

Having concluded from the previous experiments that both glyoxylic acid and formaldehyde gave coloured derivatives with tryptophan, which is confirmed by subsequent research, the nature of the reaction was next investigated. Whilst it is well known that indole and its methyl derivatives yield with different aldehydes a large number of coloured derivatives some of which have been investigated, the aldehyde reactions of tryptophan are known chiefly as qualitative colour tests and the exact nature of the compounds formed never seems to have been fully investigated. This is due partly to the difficulty of obtaining tryptophan in quantities sufficient for extended chemical research and partly to the difficulties of isolating the coloured compounds or their precursors in a stable form.

A number of colour reactions between tryptophan and various aldehydes have been described [Rohde, 1905; Sasaki, 1910], but for the purposes of the present paper it will be sufficient to consider three only: formaldehyde, glyoxylic acid and benzaldehyde. It will also be necessary to consider the reactions of these aldehydes with some substances related to tryptophan, namely: indole, scatole, and carbazole.

### Preparation and Isolation of the Aldehyde Condensation Derivatives of Indole, Scatole, Tryptophan and Carbazole.

*Condensation.* Strong sulphuric acid is quite unsuited for use as a condensing agent for these substances. It may contain a large number of active impurities; it acts as an oxidising agent in a variable manner, the heat it is apt to develop may destroy the condensation products. Fischer [1889] employed alcohol and hydrochloric acid gas for his work on the indoles. Homer and others used zinc chloride. Neither of these reagents was found to be quite satisfactory, and most of the present work was done by means of pure glacial acetic acid saturated with hydrochloric acid gas. This gave good results as a condensing agent, and is free from the objections to sulphuric acid. Phosphoric acid was used in a few particular cases. The glacial acetic acid was purified by fractionation until it no longer gave a colour with tryptophan or any of the aldehyde reagents. When pure it will keep for a long time if moisture and light are excluded, and the bottle be kept filled, as the changes leading to the formation of aldehydes appear to occur in the vapour over the liquid.

Acetic acid so prepared will be referred to in the present paper as "pure" acetic acid.

The hydrochloric acid gas for saturation was prepared by the action of pure sulphuric acid on pure dry sodium chloride. If the acid be badly contaminated with nitrous impurities some of them may pass over into the condensation mixture and spoil the preparation.

The aldehydes were used in the following forms:

Glyoxylic acid as the calcium salt. $(CHO.COO)_2 Ca, 2H_2O$.

Formaldehyde as paraformaldehyde. The ordinary solutions of "formol" are unsuitable owing to the presence of methylal and other derivatives [Werner, 1917].

Benzaldehyde as a solution in "pure" acetic acid.

One of the first facts observed during the preparation of the colour condensation compounds by the above method was that an oxidising agent was necessary in every case. The condensation products as formed are colourless or pale yellow; on oxidation the characteristic colour is produced. This fact escapes notice when sulphuric acid is used as a condensing agent since it acts as an oxidiser as well.

For the oxidation a fresh 1 % solution of $H_2O_2$ in "pure" acetic acid was used. In some instances the oxidation was allowed to proceed spontaneously in the air overnight.

*Notes on the Condensation.* It is best to use an excess of the solvent and to pass in the HCl gas slowly, shaking at intervals. After the solution has been saturated, gas is passed in for an additional quarter of an hour. A few cc. of the solution are withdrawn in a test-tube and one drop of the 1 % $H_2O_2$ added, and the tube well shaken, when the colour should develop in a few minutes. The $H_2O_2$ is added carefully up to the point of maximum depth of colour, too much will discharge the colour altogether. The approximate quantity of $H_2O_2$ is noted. Passage of HCl into the mixture is continued until no increase in depth of colour is seen on removing and testing the samples. It is sometimes useful to pass a current of air into the mixture to remove excess of HCl before the final oxidation.

The calculated amount of 1 % $H_2O_2$ is then added to the mixture, which is well shaken till oxidation is complete. It is then poured into a measuring cylinder, three times its volume of purified ether added, and the mixture shaken. The pigment will be precipitated in small flakes. If precipitation is not complete, another volume of ether may be added, but it is advisable not to exceed four volumes if a pure preparation is required, as otherwise the other substances may be precipitated.

*Isolation of the Condensation Products.* This proved to be one of the most difficult parts of the investigation. It was necessary to free the products from unchanged aldehyde, and indole derivatives, and from the condensing agent. Fractional precipitation with *pure* ether was finally adopted and gave satisfactory results. It was found that if three to four volumes of ether were added to one volume of the acetic acid solution of the pigment and the mixture well shaken, the latter came down as a flocculent precipitate and the aldehydes

and unchanged indole derivatives were left in the ethereal solution, as was also the calcium chloride which was formed when calcium glyoxylate was employed.

The purity of the ether is of great importance. It was found that merely washing and drying it was not sufficient. It should also be freed from aldehydes and related substances by distillation from sodium hydroxide, after having been left in contact with it for at least 24 hours. If the ether contains aldehydes it will react with the precipitated pigment on standing, converting it into a greenish paste, which may resinify on drying.

For this reason the coloured compounds should be filtered from the ether as soon as they are precipitated. The pyrogallol test is useful for detecting aldehydes in samples of ether.

*List of Pigments isolated.* The following are the colour derivatives obtained by the condensation of indole, scatole, tryptophan and carbazole with the three aldehydes, formaldehyde, glyoxylic acid and benzaldehyde respectively.

(The figures placed below the colour denote the number of molecules interacting.)

| Aldehyde | $CH_2O$ | CHO.COOH | $C_6H_5.CHO$ |
|---|---|---|---|
| Indole | Red<br>2I + F | Red<br>2I + G | Red<br>2I + B |
| Scatole | Red<br>2S + F | Red<br>2S + G | Purple<br>2S + B |
| Tryptophan | Red<br>2T + F | Red<br>2T + G | Blue<br>2T + B |
| Tryptophan | Blue<br>2T + 3F | Blue<br>2T + 3G | — |
| Carbazole | Blue<br>2C + F | Blue<br>2C + G | Blue<br>2C + B |

No attempt has been made to describe the shade in the above list, anything varying from rose to carmine has been grouped as "red" and similarly for the other colours. The qualitative differences are quite definite in many cases.

Many other aldehyde condensation derivatives were prepared, but the above are the most important in connection with the discussion which follows on the chemical constitution of these bodies.

*General Properties of the Condensation Derivatives.* Both the leuco-compounds and the pigments can be obtained by the ether precipitation method. The leuco-compounds are unstable and soon oxidise to form the pigments.

As precipitated the pigments are all hydrochlorides of nearly colourless bases. The pigments are soluble in strong mineral acids, in the lower aliphatic acids, in esters such as ethyl chloride, in phenol to a less extent. The solubility varies somewhat with the different pigments. The red and blue derivatives of tryptophan are hydrolysed at once when water is added to their solution in the HCl-acetic condensation mixture. The scatole and carbazole pigments are much more stable, the carbazole blues being precipitated on the addition of excess of water. Addition of alkali discharges the colour of the

pigments, as might be expected; in most cases the base is precipitated from the alkaline solution. The pigments were purified by repeated solution in "pure" acetic acid and precipitation with pure ether. They were then rapidly dried in a current of air. When pure they are amorphous deeply coloured powders.

*Melting Points.* These were all high, in the region of 300°, depending on the rate of heating. The tryptophan reds and blues tend to lose $CO_2$ on heating.

If the pigments are dissolved in absolute alcohol and treated with dry $NH_3$, they are decolorised and gradually precipitated from solution as bases, which in some cases are microcrystalline. Addition of strong acid to the bases restores the original colour of the pigments. The bases were also obtained during an attempt to crystallise the pigments from alcohol. The alcohol apparently combined with the HCl of the pigment and removed it to form ethyl chloride.

*Molecular weights.* The molecular weights of the bases were determined by the freezing point method with comparatively concordant results, using acetic acid or phenol as solvents. It has been shown that compounds of related indigotin type do not tend to form associated molecules in these solvents [Beckmann and Sabel, 1906].

The pigments are the salts of these bases.

*Analyses.* For the present preliminary work, the only element estimated was nitrogen. This was done by a modified Kjeldahl method, using mercury as catalyst instead of copper, and drawing the fumes by means of a Folin absorber into a wash-bottle, so that a control could be kept on any nitrogen which might escape combustion. These bodies can be analysed satisfactorily by the Kjeldahl method provided that the heating is continued for a considerable time after the mixture has cleared [cf. Dakin and Dudley, 1914].

The results of the nitrogen estimations were used to check the formulae of the condensation derivatives deduced from the molecular weights, sufficient tryptophan not being at my disposal for a complete analysis of the various pigments.

### Chemical Constitution of the Condensation Derivatives.

Up to the present time no structural formulae appear to have been suggested for the tryptophan pigments. From the preliminary examination of these bodies it is possible to assign formulae to them which will satisfy the requirements of the Hopkins-Cole and Rosenheim tests without attempting the more ambitious task of giving a complete account of the chemistry of these interesting condensations.

From determinations of the molecular weights of the colour bases, it appears that all the pigments prepared are the salts of compounds formed by the condensation of two molecules of the indole derivative with one of the aldehyde, except in the case of tryptophan blue, where condensation occurs with three molecules of the aldehyde.

In theory the aldehyde may unite with the indole derivative in three ways:

(1) by replacing hydrogen in the benzene ring,

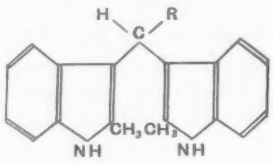
(2) by replacing hydrogen in the pyrrole ring,

(3) by replacing hydrogen in the imino-group.

The fact that all the pigments arise from oxidation of *leuco*-condensation bodies is, on the quinonoid theory of colour and constitution, strong evidence for union in either of the ways (1) or (2). The subsequent removal of hydrogen from the aldehyde residue and from one of the imino-groups occurs on oxidation and leads to the formation of the double bonds[1].

$$\underset{\text{leuco-compound}}{HN.C_6H_5—CHR—C_6H_5NH} \qquad \underset{\text{pigment}}{N=C_6H_4=CR—C_6H_5NH}$$

Fischer [1889] gives the following formula for the aldehyde derivatives of $\alpha$-methylindole (methylketole), the union taking place by the aldehyde replacing the hydrogen atoms of the $\beta$- or 3-carbon atom of the pyrrole groups.




Freund and Lebach have prepared and investigated several aldehyde derivatives of $\alpha$-methylindole (methylketole). They accept the Fischer formula, which, as they point out [1905], accounts for the production of a colour by oxidation.

Although Fischer's formula is satisfactory when applied to the aldehyde condensation derivatives of indole and methylindole, it is not so applicable to the 3-substituted indoles, such as scatole and tryptophan. Here the 3-carbon is already in union with another group and it is unlikely that under the conditions of the condensation the aldehyde displaces these groups, and even if it did, this would not explain the fact that the scatole-benzaldehyde compound is *deep purple* while the corresponding indole-benzaldehyde compound is *red*.

Again, if condensation did occur in the pyrrole ring in scatole-benzaldehyde purple, it is hard to account for the existence of the leuco-precursor. Freund and Lebach accept the formula for the aldehyde derivatives of scatole on the lines of Fischer, though they note its inability to explain the formation of the colour on oxidation.

If the aldehyde condenses with the carbons in the *benzene* ring, instead of the pyrrole ring, all these objections can be overcome. The existence of both leuco-compounds and pigments can be easily explained in the case of all the substances examined, and the general properties of the pigments can be accounted for.

The di- and tri-phenylmethane dyes in common use are quinonoid salts of bases of this type. One of their disadvantages is their sensitiveness to alkalies,

[1] The formulae are printed so as to show that the nitrogen of the imino-group is in union with two different carbon atoms.

which reconvert them into the colourless bases. It will be seen that the pigments described in the present paper have many properties in common with the di-phenylmethane dyes.

The carbazole pigments are similar in many respects to the indole derivatives. Here condensation with the aldehyde has almost certainly taken place in the benzene ring, as the pigments are produced by oxidation of leuco-compounds. The greater stability of the carbazole blues may be explained as being due to the absence of the mobile hydrogen atom of the 2-unsubstituted indoles, which is capable of changing its position from the nitrogen to the neighbouring carbon atom, and *vice versa*. The carbazole ring is stable and the imino-hydrogen is held by the presence of the benzene rings on either side. This tendency on the part of the indoles to undergo tautomeric change has been studied by various investigators [Weissgerber, 1913]. In assigning formulae to the compounds mentioned in the present paper, the hypothesis that aldehyde condensation takes place in the benzene ring has been applied. According to this, when the indole derivative reacts with the aldehyde in the presence of a condensing agent, water is eliminated and the aldehyde links up two molecules of the indole derivatives by uniting with the carbon atoms of their benzene rings to form the leuco-compound, this on oxidation loses one hydrogen atom from the aldehyde residue and one from an imino-group, with the production of the quinonoid configuration in the molecule and the formation of a colour.

These pigments, especially the benzaldehyde derivatives, and their leuco-precursors are comparable on this hypothesis with malachite green.

## Experimental.

### *The Scatole Derivatives*[1].

(1) *Scatole formaldehyde red.* $NH.C_6H_4(CH_3).CH:C_6H_4(CH_3):NX$
M.W. of base, 272.

(2) *Scatole glyoxylic red.* $NH.C_6H_4(CH_3).C(COOH):C_6H_4(CH_3):NX$
M.W. of base, 316.

(3) *Scatole benzaldehyde purple.* $NH.C_6H_4(CH_3).C(C_6H_5):C_6H_4(CH_3):NX$
M.W. of base, 348.

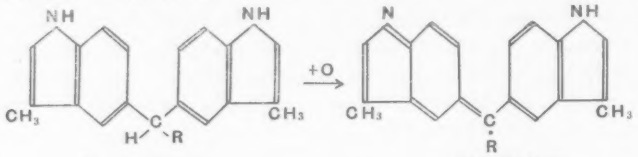
Two molecular parts of scatole and one of the aldehyde are dissolved in excess of "pure" acetic acid and condensed by means of dry hydrochloric acid gas as described. The condensation and subsequent oxidation should be done at a temperature not above 20°, otherwise the scatole itself may be oxidised with the formation of a red-brown pigment. The condensation compounds are precipitated by ether and purified as already described. They are amorphous intensely-coloured powders, fairly soluble in dilute HCl and $H_2SO_4$.

[1] The formulae are written so as to show the union of the nitrogen atom with two different carbon atoms. X represents the acid which combines with the pigment base to form the coloured salt. In the present paper X represents HCl.

The benzaldehyde derivative is the one least hydrolysed by dilution with water. They are all decolorised and precipitated by alkalies, boiling with which reconverts them in part to the original scatole. As prepared by the above method the yield is nearly quantitative.

General formula suggested for these compounds:

$$NH.C_6H_4(CH_3).CHR.\overline{C_6H_4(CH_3).NH} \xrightarrow{+O} NH.C_6H_4(CH_3).CR:\overline{C_6H_4(CH_3):NX}$$




Leuco compound — Pigment base

*The Tryptophan Derivatives*

(4) *Tryptophan formaldehyde red.* M.W. of base (average), 418.

$$CH\begin{cases}\overline{C_6H_4CH_2CH(NH_2)COOH:N}.HCl\\ \overline{C_6H_4CH_2CH(NH_2)COOH}.NH.HCl\end{cases}$$

(5) *Tryptophan glyoxylic red.* M.W. of base, above 420.

$$(COOH)\begin{cases}\overline{C_6H_4CH_2CH(NH_2)COOH:N}.HCl\\ \overline{C_6H_4CH_2CH(NH_2)COOH}.NH.HCl\end{cases}$$

(6) *Tryptophan benzaldehyde blue.* M.W. of base, 494.

$$(C_6H_5).C\begin{cases}\overline{C_6H_4CH_2CH(NH_2)COOH:N}.HCl\\ \overline{C_6H_4CH_2CH(NH_2)COOH}.NH.HCl\end{cases}$$

Two molecular parts of tryptophan are condensed with one part of the aldehyde as previously described. The preparation must be carried out in one stage, as the compounds may undergo decomposition if left for long in the condensing reagent. The temperature should not rise above 15°. If the aldehyde be added rapidly at the start in preparing tryptophan formaldehyde red, it may interact with the NH group to form a methylene derivative $—N = CH_2$, but this does not affect the general formula type given above. This methylenation of the amino-group is an intermediate stage in the formation of the blue compounds to be described later.

The tryptophan reds are the most unstable of the pigments prepared. They readily lose $CO_2$ on heating, and for this reason it is difficult to isolate tryptophan glyoxylic red, although there is evidence for its existence in solution. It is very difficult to obtain these derivatives in a pure state, apart from tryptophan benzaldehyde blue. The product from the ether precipitation appears to be a varying mixture of the true tryptophan condensation product with condensation products from decarboxylated tryptophan and tryptophan in which the amino-group has been methylenated. However, there seems to be little doubt that all these reactions are modifications of the condensation along the lines of the formulae given.

Tryptophan benzaldehyde blue is one of the more stable pigments, and seems to be well suited for colorimetric work as well as being a good reagent

for qualitative tests. Cole in 1903 drew attention to the clear colour produced. As obtained by the above method, the pigment is a deep blue powder with a faint lustre. It melts just above 310°, and rapidly decomposes according to the rate of heating. It is soluble in the solvents mentioned, but has not been crystallised from any. Like the previous tryptophan devivatives it is largely hydrolysed on diluting the acetic acid solution with water. Readings of the molecular weight of the pure base in acetic acid were on the average below 500 and just above 490, which corresponds to the theoretical value, 494, required by the formula.

(7) *Tryptophan formaldehyde blue.*
M.W., base 1, 354 (average).
M.W., base 2, 444 (average).

$$CH\begin{matrix}\nearrow C_{12}H_{10}N_2.HCl \\ \searrow C_{12}H_{11}N_2.HCl\end{matrix}$$

(8) *Tryptophan glyoxylic blue.*
M.W., variable. Above 400.

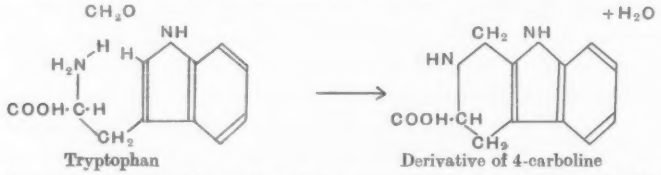
Base 1 is derived from the decarboxylated tryptophan, Base 2 from ordinary tryptophan.

*The Tryptophan Blues.* It was confirmed at an early stage in the present research that the Hopkins-Cole test and the Rosenheim test each gave *two* distinct colours with tryptophan according to the amount of aldehyde used, a fact previously noticed by Mottram [1913] in the former test. With the minimum amount of aldehyde and the test-tube kept cool, the colour in each test is *carmine*. Using more aldehyde and allowing the temperature of the mixture to rise, the colour is *violet* to *blue*. On studying the reaction using "pure" acetic acid and HCl gas, it was found that tryptophan is able to give two distinct coloured derivatives when condensed with formaldehyde or with glyoxylic acid. One class has been briefly referred to above as the tryptophan reds.

When two parts of tryptophan are condensed with three parts of formaldehyde or of glyoxylic acid at a temperature between 50° and 70°, the resulting compound on oxidation is deep blue. Also, when tryptophan red was dissolved in "pure" acetic acid saturated with HCl and left for a couple of days, it gradually changed into the blue compound. This change did not occur in acetic acid alone. The change is accompanied by the liberation of some free tryptophan, as was shown by extracting the mixture with ether containing HCl.

Investigation of the molecular weight of the HCl-free base indicates that the pigment is a di-molecular derivative of tryptophan like the other substances investigated. It was thought probable that the additional molecules of the aldehyde reacted with the *amino*-group. This was shown to be so by the preparation of some indole-hydroxypropionic acid by the action of the calculated amount of $NaNO_2$ on tryptophan in HCl solution and removal of any excess of $HNO_2$ by means of urea. This substance only gave a red aldehyde derivative under the above conditions. From this it was concluded that for the production of the *blue* pigment interaction was necessary between the aldehyde and the amino-group of the tryptophan. The first effect of such reaction would be to produce a methylene derivative of the amino-group,

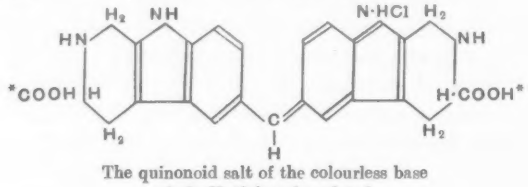
—N = $CH_2$ which would not pass into a methyl group unless the aldehyde were in considerable excess or a reducing agent were present [Werner, 1917]. Neither of these conditions obtains in the preparation as described above. It is, however, hard to understand why the methylenation of the amino-groups should change the red pigment into a blue one, and there is evidence that tryptophan red may contain such substances without alteration of colour. It is probable that there is an additional condensation. Bearing in mind the fact that the corresponding aldehyde derivatives of carbazole are all deep *blue* in colour, and that the H atom attached to the $\alpha$- or 2-carbon of indole is labile, it does not seem unreasonable to suppose that a condensation might occur between the carbon of the methylene substituting group in the tryptophan and the carbon atom of the indole group to form a three-ring molecule of the carbazole type, having a pyridine nucleus instead of one of the benzene nuclei. Such a body might be formed as follows:




Tryptophan — Derivative of 4-carboline

This ring should resemble carbazole in many of its properties. Perkin and Robinson [1919] have found a similar ring in the harmine series. They have shown that the base $C_{12}H_{10}N_2$ prepared by Hopkins and Cole [1903] by the oxidation of tryptophan with ferric chloride is harman [Fischer, 1901]. Consequently there is convincing evidence that closure of the tryptophan side-chain can occur. Perkin and Robinson advance the theory that harmine originates in nature by an acetaldehyde condensation with hydroxytryptophan, a substance already identified by Abderhalden and Baumann [1908]. Additional evidence for the closure of the tryptophan side-chain has been obtained in connection with other reactions which I hope to describe in a future communication.

On this hypothesis, the formula for tryptophan blue is:




The quinonoid salt of the colourless base
* Or H, if decarboxylated.

There appears to be very little difference between tryptophan blue prepared from formaldehyde or from glyoxylic acid. Loss of $CO_2$ occurs during the preparation and the final pigment obtained is probably the same in each case. Unless the condensation be carried out thoroughly, the pigment pre-

cipitated by the ether is very impure, containing, as well as the blue derivative, a varying amount of purple material which probably represents an earlier stage in the condensation when the side chain of only one of the tryptophan molecules has closed up. The pigment should not be precipitated until it has stood for some days, by which time most of the substances will have condensed and oxidised spontaneously to the blue.

The blue is precipitated more readily by ether than the red or the purple colouring matters, but, even so, it is almost impossible to separate them completely.

*Tryptophan formaldehyde blue* resembles the benzaldehyde blue in some respects and the carbazole blues in others.

When purified it is moderately stable and has been obtained in solution in dilute HCl. Like the other tryptophan derivatives, it melts in the region of 300°, the exact temperature depending somewhat on the rate of heating.

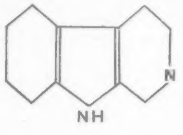
As usually carried out, the Hopkins-Cole test results in a mixture of tryptophan *red* and *blue*, the proportions depending on the conditions. For this reason it is unsatisfactory as the basis of colorimetric work, as two solutions of the same strength may give different shades if the temperature be at all different. Fasal [1912] noticed this difference in tint during his work on the estimation of tryptophan, but considered it entirely due to the amount of colouring matter formed.

For colorimetric work an aldehyde which only reacts in one way with the amino-acid is advisable, if not essential.

The above formula is in agreement with the experimental facts. It accounts for the existence of the leuco-form; for the three molecules of formaldehyde required; and for the colour and general properties of the pigment.

Only aldehydes that react readily with the amino-group, and are also capable of condensing with the indole nucleus, can give the carbolinine blue compound. Tryptophan benzaldehyde and salicyl aldehyde blues are simple condensation products and do not show any evidence of a closure of the tryptophan side-chain.

A study of the freezing-points of the bases obtained by removal of the HCl from the pigments indicated that the molecular weights of the formaldehyde and glyoxylic blues were probably the same, 352. Some preparations had higher molecular weights of the order of 400, which would be explained by the preparations not being fully decarboxylated. The pigment ultimately obtained in each case is one in which $CO_2$ has been lost by all the COOH groups. It will be seen that the condensation product formed by the closure of the tryptophan side-chain is derived from the ring nucleus:



Perkin and Robinson, who have described a similar ring, suggest the name "carboline" for this structure, indicating an analogy both to carbazole and quinoline. According to this nomenclature, tryptophan blue would be a carboline blue in which the C atoms of the pyridine nucleus are fully saturated, such a ring might be termed "carbolinine" by analogy to the relation between pyrrole and pyrroline. Tryptophan blue then would be "di-4-*iso*carbolininemethane," and the leuco-precursor would be, "di-4-carbolininemethane."

### The Carbazole Condensation Derivatives.

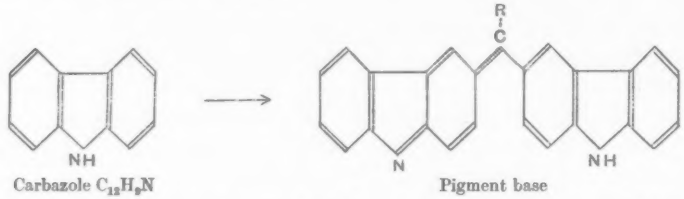
Several of these bodies were prepared: the following are of most interest in connection with the present paper.

(9) *Carbazole formaldehyde blue.*
(10) *Carbazole glyoxylic blue.*
(11) *Carbazole benzaldehyde blue.*

Only a brief reference will be made to these bodies.

Their preparation and principal properties resemble those of the pigments previously described. They are of different shades depending on the aldehyde and the solvent. They are more stable than the other pigments and will keep for a long time in aqueous solution. They are precipitated by alkalies. The melting points are high, the benzaldehyde blue beginning to decompose at 280°.

The general formula is probably:




Carbazole $C_{12}H_9N$ Pigment base

This reaction is a good general test for the presence of carbazole; benzaldehyde is perhaps the most convenient aldehyde to work with, but the colours are good with all three.

### A Simple Qualitative Reagent for Tryptophan and the Indoles.

The reagent is 10 % salicyl aldehyde in alcohol, which must be free from acetone. A small quantity of the liquid under examination is mixed with excess of strong hydrochloric acid. Four or five drops of the reagent are added and the mixture gently warmed for a few minutes, a drop of 10 % hydrogen peroxide is then added and the warming continued. An intense blue denotes tryptophan, a deep purple denotes scatole, while indole gives a bright carmine. If too much peroxide be added the colours are destroyed, otherwise, they are very stable and are not discharged on dilution with water. The reagent will

give a deep blue with less than 0·1 % tryptophan; with weak solutions care must be taken in the oxidation. By modifying the strength of the reagents the test can be made very delicate. In properties the pigments resemble very closely the benzaldehyde derivatives, their greater stability towards water is comparable with that of the malachite greens derived from substituted benzaldehyde. The fastness of these latter dyestuffs depends on the presence of a sulpho-group in the ortho-position to the methane carbon, and one is justified in assuming the hydroxyl of the salicyl aldehyde to operate in a similar manner.

Compare the behaviour of salicyl aldehyde and benzaldehyde when condensed with acetone in presence of hydrochloric acid.

## Summary.

(1) A new colour test for glyoxylic acid is described, depending on its interaction with pyrogallol in presence of sulphuric acid.

(2) The Hopkins-Cole test is due to glyoxylic acid, the Rosenheim test is due to formaldehyde. In each test at least two distinct colour derivatives of tryptophan are formed, a red and a blue.

(3) A method is described for the preparation of the aldehyde derivatives of indole, scatole, tryptophan and carbazole, and a number of these have been isolated.

(4) The possible formulae of these substances is discussed, and a general type of structure is suggested.

(5) The closure of the tryptophan side-chain is put forward as an explanation of the formation of tryptophan formaldehyde blue.

(6) Salicyl aldehyde is recommended as a useful qualitative reagent for the indole derivatives.

I should like to express my obligation to Professor F. G. Hopkins for his kind advice and interest in the research, also to Professor E. A. Werner for having read the proofs of this paper.

I received much help in the earlier stages of the work from Mr Robin Hill, of Emmanuel College, Cambridge.

The expenses of the research were defrayed by the Mackinnon Research Studentship of the Royal Society.

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# XLVII. THE HEAT INACTIVATION OF DIPHTHERIA ANTITOXIN.

By ANNIE HOMER.

(*Received May 18th, 1920.*)

Amongst serum workers there is an impression that the addition of from 1·5 to 2 % of sodium chloride to antitoxic plasma, prior to its being heated, guards against the undue inactivation of the antitoxin, and it is generally accepted that the protective influence of the sodium chloride in this respect is still more marked during the heating of cresylised plasma or serum.

In order to furnish definite evidence on this point a study has been made of the percentage inactivation of antitoxin during the heating of:

(*A*) Oxalated antitoxic plasma,

(*B*) Plasma (*A*) to which an addition of 1·5 % of solid sodium chloride was made,

(*C*) Oxalated antitoxic plasma containing 0·30 % of cresylic acid,

(*D*) Plasma (*C*) to which an addition of 1·5 % of solid sodium chloride was made,

for stated periods of time at temperatures ranging from 58° to 72°.

Separate volumes of the respective plasmas (*A*), (*B*), (*C*) and (*D*), each of 25 cc., contained in 30 cc. sealed ampoules, were heated for definite periods of time in water baths regulated to the specified temperatures by means of thermostats.

Determinations were made of the antitoxic values of the respective liquids before and after heating, and, from the results thereby obtained, was calculated the percentage inactivation induced during the heating period.

The data thus furnished from the study of the plasmas (*A*) and (*B*), and of (*C*) and (*D*), have been included in Tables I and II respectively.

It will be seen that the rate of inactivation rapidly increases with the rise in temperature. Thus (Table I), while the plasma can be kept at 58° for ten hours without suffering any appreciable diminution of its antitoxic value, there is a 10 % loss shown after three hours' heating at 60·5°, or after two hours' heating at 61·5°, or after one hour's heating at 63°. With the progressive rise in temperature the proportional loss of antitoxic value in a given time rapidly increases until at 72° there is a 60 % inactivation of the antitoxin within ten minutes.

A comparison of the results given in Tables I and II shows that the loss of antitoxin during the heating of cresylised plasma is greater than that resulting from the similar treatment of noncresylised plasma. But, contrary to the ideas current amongst serum workers, the evidence obtained in this investigation indicates that the addition of sodium chloride does not appreciably lessen the heat inactivation of antitoxin either in cresylised or in noncresylised plasma.

Further information as regards the heat-instability of antitoxin was obtained from a similar study of the inactivation of antitoxin in solutions containing only those protein fractions with which the antitoxin is associated in plasma.

The salt-soluble globulin fraction of an unheated antidiphtheritic plasma (potency 450 units per cc. and protein content 7·48 %) was separated and dialysed in the usual way. The residue from dialysis was diluted with distilled water so as to reduce the protein content of the liquid to 7 %.

This solution of antitoxin and its associated proteins, freed in this way from albumin, euglobulin and the other organic and inorganic constituents of the plasma, was submitted to the same treatment as that described above for the cresylised and noncresylised plasmas, (*A*), (*B*), (*C*) and (*D*).

The results recorded in Table III indicate that, as was anticipated, antitoxin shows a greater stability to heat when in aqueous solution associated with its own particular proteins than when present in the more complex medium of the plasma.

While studying the effect of heat on the inactivation of antitoxin I also made observations on the accompanying increased precipitability of the serum proteins. From the data, thus obtained, was calculated the percentage denaturation of the serum proteins, the results for the respective liquids being also included in Tables I, II and III.

In all cases it was noticed that, during the heating of those sera or plasmas which contained from 1·5 to 2 % of sodium chloride or cresylic acid and phenol in amounts up to 0·6 %, there was an apparently greater conversion of soluble into insoluble protein than occurred during the heating of noncresylised plasma. However, by means of the refractometer, it was ascertained that the presence of sodium chloride or of cresylic acid and phenol in the plasma had not affected the extent of the heat-denaturation of the proteins, these substances had merely stimulated the coagulation of the particles of denaturated proteins into aggregates of sufficient size to be more readily discerned by the eye.

From the results recorded both here and in my previous papers it has been found that the extent of the denaturation of the proteins is a function of:

(*a*) the temperature at which the heating was conducted;

(*b*) the reaction of the plasma;

and (*c*) the duration of the heating of the plasma.

Table I.

*Showing the extent of the denaturation of the serum proteins and the inactivation of the antitoxin during the heating of oxalated antidiphtheritic plasma* (A) *and of the same plasma to which has been added* 1·5 % *of sodium chloride* (B).

Potency of the unheated plasma = 375 units per cc.
Protein-content of the plasma = 8·45 %
$P_H$ of the unheated plasma, 7·8.

| | | Plasma (*A*) | | Plasma containing 1·5 % NaCl (*B*) | |
|---|---|---|---|---|---|
| Temperature °C. | Duration of the heating | Percentage denaturation of the serum proteins | Percentage inactivation of the antitoxin | Percentage denaturation of the serum proteins | Percentage inactivation of antitoxin |
| 58 | 4·5 hours | 30·3 | nil | 30·5 | nil |
| ,, | 10 ,, | 31·0 | ,, | 31·2 | ,, |
| 60·5 | 1 hour | 13·5 | ,, | 14·0 | ,, |
| ,, | 2 hours | 40·0 | ,, | 41·0 | ,, |
| ,, | 3 ,, | 42·0 | 10 | 41·5 | less than 10 |
| 61·5–62 | 1·5 ,, | 40·0 | — | 40·0 | — |
| ,, | 2 ,, | 50·0 | 8 | 49·0 | less than 8 |
| ,, | 3 ,, | 55·0 | slightly greater than 13 | 55·0 | about 20 |
| ,, | 4 ,, | 56·5 | | 57·5 | |
| 63 | 1 hour | 52·0 | 10 | 53·5 | 10 |
| ,, | 2 hours | 64·0 | slightly less than 27 | 66·0 | 25 |
| ,, | 3 ,, | 72·0 | — | 70·0 | — |
| 65 | 10 minutes | 40·8 | — | 42·5 | — |
| ,, | 20 ,, | 57·0 | about 27 | 58·5 | about 27 |
| ,, | 30 ,, | 65·0 | about 33 | 66·0 | about 33 |
| ,, | 1 hour | practically solid | — | practically solid | — |
| 66 | 10 minutes | 44·5 | about 13 | 45·5 | about 10 |
| ,, | 20 ,, | 60·0 | slightly less than 33 | 62·0 | slightly less than 33 |
| 67·5 | 10 ,, | 62·5 | 20 | 63·5 | 20 |
| ,, | 20 ,, | 69·0 | 36 | 71·0 | 33 |
| 68·5–69 | 5 ,, | 56·5 | — | 57·0 | — |
| ,, | 10 ,, | 62·0 | 33 | 61·0 | 33 |
| ,, | 20 ,, | 72·0 | slightly less than 46 | 72·0 | slightly les than 46 |
| 70 | 5 ,, | 65·0 | slightly less than 33 | 65·0 | 33 |
| ,, | 10 ,, | 75·0 | 53 | 75·0 | slightly greater than 46 |
| ,, | 15 ,, | almost sclid | — | practically solid | — |
| 72 | 5 ,, | 80·0 | 60 | 80·0 | 60 |
| ,, | 10 ,, | practically solid | — | practically solid | — |

Table II.

*Showing the extent of the denaturation of the serum proteins and the inactivation of antitoxin induced during the heating of antidiphtheritic plasma containing* 0·30 % *of cresylic acid* (C), *and of the same cresylised plasma to which* 1·5 % *of sodium chloride has been added* (D).

Potency of the original plasma = 375 units per cc.

$P_H$ of the original plasma, 7·4.

Protein-content of the plasma = 8·45 %.

| Temperature °C. | Duration of the heating | Cresylised plasma (*C*) | | Cresylised plasma containing 1·5 % of NaCl (*D*) | |
|---|---|---|---|---|---|
| | | Percentage denaturation of the serum proteins | Percentage inactivation of the antitoxin | Percentage denaturation of the serum proteins | Percentage inactivation of the antitoxin |
| 58 | 4 hours | 25·0 | nil | 25·4 | nil |
| „ | 8 „ | 26·0 | „ | 26·0 | „ |
| 60 | 1 hour | 41·6 | negligible | 40·6 | negligible |
| „ | 2 „ | 45·5 | 10 | 45·4 | 10 |
| „ | 3 „ | 48·5 | 17 | 48·3 | 17 |
| 62 | 1 „ | 45·0 | 17 | 47·2 | 13 |
| „ | 2 hours | 55·0 | — | 57·5 | 24 |
| „ | 3 „ | 57·0 | — | 57·5 | — |
| 63 | 1 hour | 47·2 | 17 | 50·0 | 17 |
| „ | 2 hours | 64·0 | — | 65·0 | — |
| „ | 3 „ | 70·0 | — | practically solid | — |
| 64 | 30 minutes | 53·0 | 15 | 52·0 | 17 |
| „ | 1 hour | 63·5 | — | 66·0 | — |
| „ | 2 hours | practically solid | — | practically solid | — |
| 65 | 20 minutes | 53·0 | 40 | 52·0 | 40 |
| „ | 30 „ | 65·0 | — | 64·0 | — |

The extent of the inactivation of the antitoxin seems to be regulated by:

(*a*) the temperature at which the heating is conducted;

and (*b*) the time of heating.

The time of heating required to produce the maximum value for the denaturation of the proteins became shorter as the temperature was raised. In this respect the behaviour of antitoxin and of the serum proteins is somewhat analogous. However the heat-inactivation of the antitoxin seems to be mainly a function of the temperature and independent of the extent of the accompanying heat-denaturation of the serum proteins, for, by suitable adjustments, the heat-denaturation of the proteins at a given temperature can be rendered complete without any corresponding change in the coefficient for the heat-inactivation of the antitoxin at that temperature.

These observations lead to considerations as to whether antitoxins should be regarded as separate entities rather than as structural modifications of the serum proteins induced during the process of immunisation. In this connection, it had been my intention to plot curves for the time taken, at each degree of temperature, to reduce the antitoxic value of the liquids to half

Table III.

*Showing the extent of the heat-denaturation of pseudo-globulin and of the inactivation of antitoxin in solutions which have been heated at temperatures between 58° and 72°.*

$P_H$ of the solution of pseudo-globulin, 7·0.

Potency = 450 units per cc.; protein-content, 7·48 %.

| Temperature °C. | Duration of the heating | Percentage denaturation of the pseudo-globulin | Percentage inactivation of antitoxin |
|---|---|---|---|
| 58 | 4 hours | 25·0 | negligible |
| „ | 10 „ | 26·0 | „ |
| 61·5 | 1 hour | 23·7 | nil |
| „ | 2 hours | 26·6 | negligible |
| „ | 3 „ | 31·6 | „ |
| 63 | 30 minutes | 31·0 | „ |
| „ | 1 hour | 40·0 | „ |
| „ | 2 hours | 51·0 | „ |
| „ | 3 „ | 55·0 | „ |
| 66 | 20 minutes | 40·0 | „ |
| „ | 40 „ | 46·0 | „ |
| 67·5 | 10 „ | 50·7 | „ |
| „ | 20 „ | 61·7 | nearly 13 |
| 68·5 | 10 „ | 57·0 | 8 |
| „ | 20 „ | 67·0 | — |
| 70·0 | 5 „ | 50 | negligible |
| „ | 10 „ | 70 | 30 |
| „ | 15 „ | practically solid | — |
| 72 | 8 „ | 90 | greater than 50 |

value, and to make therefrom a comparison between the heat inactivation of antitoxin and that of certain enzymes. But, unfortunately, owing to the prolonged scarcity of experimental animals, I have been unable to carry my investigations further in this respect.

The work involved in this investigation was, in part, carried out during my war time appointment with the Lister Institute.

## Summary.

During the course of the investigation it has been found that:

(1) The addition of 1·5 to 2 % of sodium chloride to plasma, whether noncresylised or cresylised, prior to its being heated, in no way reduces the extent of the heat-inactivation of the antitoxin.

(2) The rate of the heat-inactivation of antitoxin is a function of the temperature at which the heating is conducted.

(3) The extent of the heat-inactivation of the antitoxin in an aqueous solution of the antitoxin bearing proteins is considerably less than that evidenced during the heating of antitoxic plasma.

# XLVIII. NOTE ON "SCURVY" IN PIGS.

By ROBERT HENRY ADERS PLIMMER.

*From the Rowett Research Institute in Animal Nutrition, University of Aberdeen and North of Scotland College of Agriculture.*

(*Received May 19th, 1920.*)

At the beginning of April four young pigs were observed to be ill with symptoms suggesting scurvy, namely:

(1) Stoppage of growth.

(2) On being approached the animals squealed with more than their usual fervour, and on being touched the squealing became frenzied.

(3) Lack of appetite and a condition of great lassitude.

(4) Spasmodic twitching of limb muscles (see description of scurvy in guinea-pigs by Miss Chick and co-workers).

(5) Swollen joints.

(6) Inability to walk or stand properly. On being made to rise or move, the pigs dragged themselves about with the trotters bent underneath the forelimbs. The hind limbs were also feeble and not held straight as in the normal pig, but the trotters were not bent under as in the forelegs.

(7) The gums and teeth could not be examined, as the animals resented being touched and were likely to bite.

On enquiry into the diet it was ascertained that the pigs had been fed entirely on *cooked* food, a mash composed of meal, sharps and turnips. As it seemed obvious that the complaint was due to lack of the antiscorbutic factor, directions were given that the same food should be continued but in a *raw* state. The proportion of turnip (yellow swede) was increased in amount because of the high antiscorbutic value which this vegetable is known to possess. In this case the swedes were obtained direct from the field in which they were growing. Occasionally a little skimmed milk or butter milk was given both with the cooked and the raw food at those times when it was available on the farm.

As was expected the four pigs very soon improved upon the uncooked diet; their appetite showed improvement after a few meals of raw food. The other symptoms gradually lessened until in about 14 days the pig in the best condition roamed about the fields of his own free will, and on handling squealed no more than is customary with pigs. The other three pigs, whose symptoms had been more acute, took five weeks to reach the same state of recovery. The best pig walked properly after a week. The others walked on

straight limbs after four weeks, and their limbs, though still weak, are gradually gaining strength.

The animals had the following weights in kilograms which show their improved condition:

| | April 9th | April 20th | April 27th | May 17th |
|---|---|---|---|---|
| (1) | 25 | 32 | 34·5 | 41·6 |
| (2) | 25 | 29·4 | 35 | 44·5 |
| (3) | 38 | 37·4 | 41·5 | 50·5 |
| (4) | 20·7 | 22·8 | 25·5 | 31·5 |

The change to uncooked food has thus not only improved their condition, but has caused renewed growth. The animals were about four months old at the time they first came under observation. No. 3 took longer than the others to recover its appetite.

This case exemplifies the application of recent scientific results to agricultural problems; with the absence of suitable appliances for scientific work it was not possible to make closer study such as weighing of the food and more details of the symptoms of the animals. This disease is said to be fairly common amongst pigs reared during the winter and fed on cooked food as well as being much confined to styes. It is most frequently referred to as "rheumatism," sometimes as "rickets" or "pig gout"; raw potatoes are in some places recommended for its cure. The term rickets as here applied is evidently incorrect, since the antirachitic factor is comparatively stable to heat. Delf [1920] has shown that cooking rapidly destroys the antiscorbutic factor.

*Addendum, August 11th*, 1920.

Since the above note was written, the pigs have been sold on the market and realised the current prices. Fortunately it was possible to see the ribs and bones of pig (4). The ribs on one side appeared normal: on the other side one rib showed a healed fracture (of old standing) and five other ribs showed haemorrhage; two of these ribs were curved and not straight. The leg bones of one side looked normal: those of the other side showed (*a*) thickening of the radius and ulna, (*b*) fusion and thickening of the tibia and fibula.

REFERENCE.

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# XLIX. NOTE ON THE OXIDATION OF QUININE WITH HYDROGEN PEROXIDE.

By MAXIMILIAN NIERENSTEIN.

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(*Received May 19th, 1920.*)

The observations recorded in this note refer to experiments which were carried out in the hope of oxidising quinine to haemoquinic acid, a disintegration product of quinine found in the urine, especially in blackwater fever [Nierenstein, 1918, 1919]. The results were disappointing, since it was only possible to identify quitenine, a well-known oxidation product of quinine, though a very careful search was made for haemoquinic acid. In this connection it is of interest to note that quitenine is also present in the urine after the administration of quinine [Nierenstein, 1918, 1919] and that it is produced from quinine by emulsions from guinea-pig and ox liver [Lipkin, 1919].

Ten grams of quinine hydrochloride dissolved in 100 cc. of water were heated on a water-bath for 6 hours with 50 cc. hydrogen peroxide (40 volumes commercial) and the oxidation repeated with a fresh amount of hydrogen peroxide (50 cc.) for a further 6 hours. To the solution, which had acquired a deep, dark-brown colour, the theoretical amount of $N/10$ sodium hydroxide was added, so as to neutralise the hydrochloric acid present. The solution, which contained a fair amount of a precipitate, was extracted several times with ether, and the ethereal extract shaken with a 10 % solution of sodium bicarbonate saturated with carbon dioxide. The aqueous layer was acidified and extracted with ether. The ethereal extract, carefully dried over anhydrous sodium sulphate, left on evaporation a residue which crystallised from dilute alcohol in slender needles, melting at 280–282° when quickly heated, carbon dioxide being evolved. This melting point was not depressed when mixed with quitenine prepared according to Skraup's method [1893]. The melting point for quitenine is given by Skraup as 286° and by Nierenstein [1919] as 283–284°. Three different preparations, dried at 150°, were used for analysis.

0·1384 g.: 10·0 cc. $N_2$ at 11° and 753 mm.
0·1562 g.: 11·2 cc. $N_2$ at 15° and 757 mm.
0·1614 g.: 11·5 cc. $N_2$ at 15° and 747 mm.

Found: N = 8·6; 8·3; 8·2.

Calculated for $C_{19}H_{22}O_4N_2$: N = 8·2 %.

The quitenene prepared both by the hydrogen peroxide method and according to Skraup's method gave the thalleioquin reactions described by Lipkin [1919], which confirms his very exact observations.

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# L. GLYCINE AND ITS NEUTRAL SALT ADDITION COMPOUNDS.

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(*Received May 26th, 1920.*)

The work of Bugarsky and Liebermann [1898] showed that proteins could combine with acids and alkalies but not with sodium chloride. It is not however universally accepted that no combination takes place between proteins and neutral salts in general. The behaviour of amino-acids, as forming the fundamental units of the protein molecule, towards neutral salts is therefore of some interest.

Scattered throughout the early literature, there are several references to the addition compounds of glycine and inorganic salts. It is, however, to Pfeiffer and his co-workers that we are indebted for a fuller and more systematic account of the addition products of neutral salts, particularly the halides of the alkali and alkaline earth metals with glycine, its simpler derivatives, and alanine.

By mixing glycine and the chlorides or bromides of the alkaline earth metals or of lithium in suitable proportions in aqueous solution Pfeiffer and Modelski [1912, 1913] obtained on evaporating the solution, or on adding alcohol, crystalline addition complexes, containing the components in some simple molecular proportion. Thus calcium chloride was found to furnish three compounds, $CaCl_2, C_2H_5O_2N, 3H_2O$; $CaCl_2, (C_2H_5O_2N)_2, 4H_2O$, and $CaCl_2, (C_2H_5O_2N)_3$ and lithium bromide two compounds, $LiBr, C_2H_5O_2N, H_2O$, and $LiBr, (C_2H_5O_2N)_2, H_2O$. The formation and isolation of these pure substances were materially assisted by the addition to the clear solution of a little acetic acid. Bayliss [1915, 1919, 2] has on several occasions taken exception to the description of these addition products as compounds, his own preliminary experiments having indicated that the problem was more complex and involved the formation of mixed crystals.

One of the present authors, having had experience of addition compounds of the alkaline earth halides and also of the alkali metal halides with an amphoteric substance of quite a different type, it was decided to attempt to throw further light on these addition compounds of glycine under such conditions as would lead to results of significance. The addition of acetic acid

and precipitation by alcohol were therefore avoided throughout. We have, moreover, restricted the experiments to solutions containing one molecular proportion of glycine in the case of lithium, sodium and potassium chlorides, bromides and iodides, and two molecular proportions in the case of calcium strontium and barium chlorides and iodides, except where the behaviour of such a solution rendered it necessary to start with different proportions of the components.

In the main we confirm the results of Pfeiffer and Modelski with regard to the definite nature of the compounds of glycine with calcium, barium and strontium chlorides, of the type $MCl_2, (C_2H_5O_2N)_2, H_2O$, and with lithium chloride and bromide of the types, LiCl (or Br), $C_2H_5O_2N, H_2O$ and LiCl (or Br), $(C_2H_5O_2N)_2, H_2O$. We have been unable to prepare any addition compounds of the potassium halides, but have succeeded in preparing from sodium bromide and iodide, addition compounds of the same type as the second lithium type figured above. We are also able to support Bayliss's contention of the formation of solid solutions, which in our opinion are formed by the mutual solubility of the addition compounds in one another.

The different degrees of complexity of behaviour over the somewhat restricted range of composition of the solutions as studied by us are shown by the following examples.

Barium chloride and glycine form a compound, $BaCl_2, (C_2H_5O_2N)_2, H_2O$, which can be obtained pure from a solution containing the components in widely differing molecular proportions.

| Ratio $BaCl_2/C_2H_5O_2N$ | 1 : 2 | 3 : 4 | 1 : 4 |
|---|---|---|---|
| % yield of $BaCl_2, (C_2H_5O_2N)_2, H_2O$ | 60 | 64 | 48 |
| Analysis | Cl = 18·8 | Cl = 18·8 | Cl = 18·7 |
| | N = 7·6 | N = 7·5 | N = 7·7 |

Calculated for $BaCl_2, (C_2H_5O_2N)_2, H_2O$; Cl = 18·8; N = 7·4 %.

This table furnishes a proof of the true molecular compound nature of these addition products. Moreover, the water of crystallisation present in all of these addition compounds is in some simple molecular proportion and is not the same as is present in the pure inorganic constituent, when in equilibrium with its saturated solution at the ordinary room temperature.

The behaviour of lithium bromide and glycine is of interest as it affords evidence that perfect homogeneity of crystalline appearance is not in itself a sufficient criterion of a definite chemical compound. A solution of these components in molecular proportions deposits on concentration first well-formed transparent needles, which are soon followed by a voluminous deposit of smaller white needles. If the transparent needles be collected before the second type has appeared, their Br/N ratio is approximately 0·87/1. This ratio is intermediate between the two compounds, LiBr, $C_2H_5O_2N, H_2O$ and LiBr, $(C_2H_5O_2N)_2, H_2O$, which were obtained by Pfeiffer and Modelski and have also been obtained by us, and the crystals represent a solid solution of the one compound in the other.

The iodides of calcium, strontium and barium when crystallised from a solution containing two molecular proportions of glycine do not give pure compounds. The crystals are generally poor in appearance when compared with the corresponding chlorides, and probably represent solid solutions of more or less complete miscibility. From the mother liquor of such a calcium iodide—glycine solution, which, having already deposited crystals whose I/N ratio was 0·57/1, was relatively richer in calcium iodide, the most markedly crystalline compound yet encountered in these experiments was obtained. It crystallised in large tablets of the composition, $CaI_2$, $(C_2H_5O_2N)_2$, $3H_2O$. These were shown to be stable only in contact with solutions containing excess of calcium iodide. This behaviour is strictly analogous to the well-known instability of carnallite, KCl, $MgCl_2$, $6H_2O$ except in contact with solutions containing excess of magnesium chloride.

Lithium iodide and glycine in molecular proportions did not furnish a pure homogeneous compound.

From aqueous glycine solutions or from solutions of glycine containing neutral salts, we have repeatedly isolated an unstable form of glycine crystallising in long stout needles or columns. On keeping in contact with the solution these gradually disappear with simultaneous deposition of the oft-described and pictured, squat monoclinic crystals [Schmelcher, 1892]. Curtius [1882] seems to have observed the formation of these two kinds of crystal from aqueous solution, but did not recognise their relative stability. The remarkable experiments of Ostromisslenski [1908] on the inoculation of *dl*-asparagine solutions with glycine, to explain which he postulates the existence of two enantiomorphous forms of glycine and moreover states that glycine crystallises in the rhombic hemihedral system and not in the monoclinic, may be intimately connected with the existence of the two forms observed by us. Fischer [1905] obtained a form of glycine which was reactive towards phosphorus pentachloride by precipitating its aqueous solution with alcohol. This form we consider to be probably identical with our unstable form from aqueous solutions. Falk and Sugiura [1918], repeating Fischer's preparation, showed that this reactive form, precipitated by alcohol in the form of fine needles, differed from the ordinary form in its greater absorption of bromine in the dry state and in its slightly slower evolution of gas in the determination of the van Slyke values. The difference in behaviour in the region of the melting point as described by these authors we have shown to be independent of any difference of structure. To account for these two glycines they suggest as a possibility a difference of structure, $NH_2.CH_2.COOH$ and $\overline{NH_3.CH_2.COO}$.

It is of interest that in reviewing the nature of the linking of affinities in these addition compounds, Pfeiffer and Wittka [1915] reject the view expressed in the earlier paper of Pfeiffer and Modelski that the addition compounds were amphi-salts of the type $LiOOC.CH_2.NH_2.HCl$ and now suppose that

the metal portion of the inorganic salt is attached to the closed ring form of glycine by means of the residual affinity of the carbonyl group, *e.g.* ClM... $(\overline{OOC.CH_2NH_3})_2$.

## Experimental Details.

*Glycine Addition Compounds of Calcium, Strontium and Barium Chlorides.*

*Barium Chloride.* 5 grams of glycine ester hydrochloride were boiled for about 45 minutes with 20 cc. of water and 5 g. of barium carbonate. The solution was then filtered and on concentration yielded well-formed transparent plates. The first and second crops possessed a perfectly homogeneous appearance and amounted respectively to 2·3 and 1·2 g. The air-dried substance was analysed.

0·2033; 0·1557 g. AgCl. Cl = 18·9 %.
0·2982; $NH_3$ equivalent to 16·08 cc. *N*/10 acid. N = 7·6 %
0·1981 dried at 120° lost 0·0103 g. $H_2O$ = 5·2 %.
Calculated for $BaCl_2(C_2H_5O_2N)_2, H_2O$; Cl = 18·8; N = 7·4; $H_2O$ = 4·8 %.

The same compound was obtained from a mixture of one molecule of barium chloride and two molecules of glycine. Thus 1 g. of glycine and 13·9 cc. of 10 % barium chloride solution gave on concentration 1·5 g. (60 % yield) of addition compound crystallising in stout needles.

0·2092 g. air-dry; 0·1586 g. AgCl. Cl = 18·8 %.
0·2089 g. air-dry; $NH_3$ equivalent to 11·28 cc. *N*/10 acid. N = 7·6 %.

When the proportion of barium chloride was increased to three molecules to four of glycine, the same compound was isolated pure. One gram of glycine gave 1·6 g. (64 % yield) of addition compound.

0·2045 g. air-dry; 0·1557 g. AgCl. Cl = 18·8 %.
0·2045 g. air-dry; $NH_3$ equivalent to 11·01 cc. *N*/10 acid. N = 7·5 %.

When, however, the proportion of barium chloride was decreased to one molecule to four molecules of glycine, crystals of the addition compound separated followed by free glycine. By filtration before the glycine crystals began to appear, 1·2 g. (48 % yield) of pure addition compound were obtained from 2 g. of glycine.

0·2017 g. air-dry; 0·1522 g. AgCl. Cl = 18·7 %.
0·2021 g.; $NH_3$ equivalent to 11·06 cc. *N*/10 acid. N = 7·7 %.

*Calcium Chloride.* When the preparation of the calcium and strontium addition compounds of glycine was attempted starting with glycine ester hydrochloride and excess of calcium or strontium carbonates it was unexpectedly found that after prolonged boiling there was only slight decomposition of the ester hydrochloride, for on concentrating, the first crop of crystals consisted in each case of pure glycine ester hydrochloride, crystallising in the characteristic long silky needles, melting about 140° and analysing correctly. (Found Cl = 25·3; N = 10·0; 9·9; $C_4H_9O_2N$, HCl requires Cl = 25·4; N = 10·0 %.)

The required addition compound was readily obtained by boiling the ester hydrochloride of glycine with excess of halogen-free slaked lime, filtering, saturating with carbon dioxide and further boiling to decompose the bicarbonate and carbamate formed. The calcium chloride addition compound crystallised in prismatic needles.

0·1995 g. air-dry; 0·1700 g. AgCl. Cl = 21·1 %.
0·2023 g. air-dry; $NH_3$ equivalent to 12·06 cc. *N*/10 acid. N = 8·3 %.
Calculated for $CaCl_2$, $(C_2H_5O_2N)_2$, $4H_2O$; Cl = 21·3; N = 8·4 %.

*Strontium Chloride.* The strontium chloride addition compound crystallised similarly in prismatic needles.

0·2044 g. air-dry; 0·1573 g. AgCl. Cl = 19·0 %.
0·2030 g. air-dry; $NH_3$ equivalent to 11·12 cc. *N*/10 acid. N = 7·7 %.
Calculated for $SrCl_2$, $(C_2H_5O_2N)_2$, $3H_2O$; Cl = 19·5; N = 7·7 %.

The somewhat divergent chlorine value was accidental, for, on recrystallisation, the compound gave the following figures on analysis.

0·2053 g. air-dry; 0·1613 g. AgCl. Cl = 19·4 %.
0·2033 g. air-dry; $NH_3$ equivalent to 11·10 cc. *N*/10 acid. N = 7·6 %.
0·2381 g. dried at 140° lost 0·0356 g. $H_2O$ = 15·0 %. (Theory requires 14·9 % $H_2O$.)

*Glycine Addition Compounds of Calcium, Strontium and Barium Iodides.*

*Barium Iodide.* An aqueous solution of two molecular proportions of glycine to one of barium iodide was concentrated under reduced pressure to a small volume. It deposited, when cold, a felted mass of woolly needles. On analysis the I/N ratio showed it to be a mixture.

0·2049 g. air-dry; 0·1508 g. AgI. I = 39·8 %.
0·2054 g. air-dry; $NH_3$ equivalent to 9·8 cc. *N*/10 acid. N = 6·7 %.
Whence I/N = 0·65/1.

*Strontium Iodide.* Glycine (two molecular proportions) and strontium iodide gave a felted mass of crystals. Analysis indicated a mixture.

0·2031 g. air-dry; 0·1496 AgI. I = 39·8 %.
0·2073 g. air-dry; $NH_3$ equivalent to 12·46 cc. *N*/10 acid. N = 8·4 %.
Whence I/N = 0·52/1.

*Calcium Iodide.* Glycine and calcium iodide were mixed in aqueous solution in the proportion of two molecules of glycine to one of calcium iodide. On concentration under reduced pressure and keeping at room temperature clusters of small needles were deposited. These were collected, air-dried and analysed.

0·2029 g.; 0·1674 g. AgI. I = 44·6 %.
0·2013 g.; $NH_3$ equivalent to 12·4 cc. *N*/10 acid. N = 8·6 %.
Whence I/N = 0·57/1.

This sample was recrystallised from water and separated in small needles. The air-dry substance was analysed.

0·2021 g.; 0·1603 g. AgI. I = 42·9 %.
0·2013 g.; $NH_3$ equivalent to 13·96 cc. *N*/10 acid. N = 9·7 %.
Whence I/N = 0·49/1.

In spite of the apparently satisfactory atomic ratio the crystals do not, probably, represent a pure individual compound.

The first mother liquors, after further concentration, deposited a felted mass of needles, but after standing for three or four weeks these had almost completely dissolved with deposition of large clear tablets. These were carefully picked out and analysed.

0·2040 g. air-dry; 0·1922 g. AgI. I = 50·9 %.
0·2063 g. air-dry; $NH_3$ equivalent to 8·55 cc. *N*/10 acid. N = 5·8 %.
Calculated for $CaI_2$, $(C_2H_5O_2N)_2$, $3H_2O$; I = 51·0; N = 5·6 %.

On keeping overnight the filtrate gave a further deposit of well-formed smaller tablets. These on analysis proved to be the same compound.

0·2010 g. air-dry; 0·1890 AgI. I = 50·8 %.
0·2084 g. air-dry; $NH_3$ equivalent to 8·55 cc. *N*/10 acid. N = 5·7 %.

With these crystals in hand a fresh calcium iodide solution containing two molecular proportions of glycine was prepared and concentrated. In spite of numerous attempts, inoculation with this well-formed crystalline trihydrate failed to cause any further separation of this form, the solution invariably yielding small needles. Nor did these change on prolonged keeping.

When, however, a solution of molecular proportions of glycine and calcium iodide was concentrated, inoculation with the trihydrate caused immediate separation of this compound.

0·2058 g. air-dry; 0·1919 AgI. I = 50·4 %.
0·2046 g. air-dry; $NH_3$ equivalent to 8·44 cc. *N*/10 acid. N = 5·8 %.

This compound is therefore only stable at room temperature in contact with solutions containing excess of calcium iodide.

*Glycine Addition Compounds of the Lithium Halides.*

*Lithium Chloride.* Glycine ester hydrochloride was boiled with an aqueous solution of one molecular proportion of lithium carbonate. On concentration the solution deposited, when cold, well-formed prismatic needles. When crystallisation of this form was almost complete a few crystals, smaller and opaque white in appearance, separated. The proportion of this latter was, however, quite small, and its effect is shown by the analysis of the total solid separation.

0·2059 g. air-dry; 0·2094 g. AgCl. Cl = 25·2 %.
0·2034 g. air-dry; 0·2073 g. AgCl. Cl = 25·2 %.
0·2015 g. air-dry; $NH_3$ equivalent to 15·61 cc. *N*/10 acid. N = 10·8 %.
Calculated for LiCl, $C_2H_5O_2N$, $H_2O$; Cl = 26·2; N = 10·3 %.

It was recrystallised and separated in transparent prismatic needles of perfectly uniform appearance.

0·2013 g.; 0·2114 g. AgCl. Cl = 26·0 %.
0·2073 g.; $NH_3$ equivalent to 15·53 cc. *N*/10 acid. N = 10·5 %.

*Lithium Bromide.* A concentrated solution of molecular proportions of glycine and lithium bromide deposited first a crop of moderately long transparent needles, but before crystallisation was complete, a voluminous mass

of fine white needles separated as well. The total crop was collected and analysed when air-dry.

0·2021 g.; 0·1477 g. AgBr. Br = 31·1 %.
0·2010 g.; $NH_3$ equivalent to 14·38 cc. *N*/10 acid. N = 10·2 %.
Calculated for LiBr, $(C_2H_5O_2N)_2$, $H_2O$. Br = 31·3; N = 11·0 %.

A repetition of this experiment reproduced an identical behaviour.

Found on analysis, Br = 32·9; N = 10·6 %.

The experiment was now repeated, but the first crop of transparent needles, possessing all the apparent attributes of homogeneity, was collected before the second kind of crystal had begun to separate.

0·1767 g. air-dry; 0·1721 AgBr. Br = 41·4 %.
0·1571 g. air-dry; $NH_3$ equivalent to 9·37 cc. *N*/10 acid. N = 8·4 %.
Whence Br/N = 0·87/1.

Two repetitions of this preparation in which the first, apparently homogeneous, separation of crystals was collected, gave similar figures on analysis.

Found Br = 41·4; N = 8·5. Whence Br/N = 0·85/1.
Found Br = 42·2; N = 8·4. Whence Br/N = 0·88/1.

In the presence of one-half a molecular proportion of lithium bromide in excess, a uniform crop of transparent prismatic needles was obtained. These were collected and analysed.

0·1943 g. air-dry; 0·2015 g. AgBr. Br = 44·1 %.
0·2023 g. air-dry; $NH_3$ equivalent to 10·92 cc. *N*/10 acid. N = 7·6 %.
Calculated for LiBr, $C_2H_5O_2N$, $H_2O$. Br = 44·4; N = 7·8 %.

When the proportion of lithium bromide was reduced to three-fourths of a molecule, a crop of crystals was obtained possessing a homogeneous appearance and crystallising in transparent needles. This result was not obtained without some difficulty as the solution had to be heated up and allowed to crystallise several times before what were undoubtedly uniform crystals were obtained.

0·2001 g. air-dry; 0·1489 g. AgBr. Br = 31·7 %.
0·2005 g. air-dry; $NH_3$ equivalent to 15·71 cc. *N*/10 acid. N = 11·0 %.
Calculated for LiBr, $(C_2H_5O_2N)_2$, $H_2O$. Br = 31·3; N = 11·0 %.

When the proportion of lithium bromide was lowered still further, to one-half a molecule, it was not found possible to obtain a crop of crystals of homogeneous appearance. Pfeiffer and Modelski, using these proportions, admittedly after some difficulty, obtained tablets of the compound LiBr, $(C_2H_5O_2N)_2$, $H_2O$, which we obtained as described above, from a solution somewhat richer in glycine, but crystallising in needles.

*Lithium Iodide.* From a solution of molecular proportions of glycine and lithium iodide, clusters of fine white silky needles separated, not unlike the mixed crystals obtained from glycine and the iodides of the alkaline earth metals.

0·2017 g. air-dry; 0·1980 g. AgI. I = 53·1 %.
0·2028 g. air-dry; $NH_3$ equivalent to 11·01 cc. *N*/10 acid. N = 7·6 %.
Whence I/N = 0·77/1.

A second experimental solution of molecular proportions was prepared and several attempts made to obtain uniform crystals of a different type, but without success.

*Glycine Addition Compounds of the Sodium Halides.*

*Sodium Bromide.* Glycine and sodium bromide in molecular proportions gave, on concentrating the solution, a homogeneous crop of well-defined transparent needles. These were collected and analysed.

0·2016 g.; 0·1442 g. AgBr. Br = 30·4 %.
0·2023 g.; $NH_3$ equivalent to 15·09 cc. *N*/10 acid. N = 10·4 %.
Calculated for NaBr, $(C_2H_5O_2N)_2$, $H_2O$. Br = 29·5; N = 10·3 %.

A second solution was prepared with the same proportions, but considerable difficulty was experienced in obtaining a crop of crystals of homogeneous appearance. On inoculation, however, with the compound obtained in the first experiment, the same compound, crystallising in well-defined transparent needles, was obtained as before.

0·1562 g. air-dry; 0·1079 AgBr. Br = 29·4 %.
0·1695 g. air-dry; $NH_3$ equivalent to 12·65 cc. *N*/10 acid. N = 10·4 %.

*Sodium Iodide.* Glycine and sodium iodide in solution in molecular proportions gave, without difficulty, a crop of well-defined needles grouped in clusters.

0·2025 g. air-dry; 0·1501 g. AgI. I = 40·0 %.
0·2007 g. air-dry; $NH_3$ equivalent to 12·63 cc. *N*/10 acid. N = 8·8 %.
Calculated for NaI, $(C_2H_5O_2N)_2$, $H_2O$. I = 39·9; N = 8·8 %.

A second solution of the same initial composition was prepared and on concentration readily gave the same compound.

0·1983 g.; 0·1463 g. AgI. I = 39·9 %.
0·2028 g.; $NH_3$ equivalent to 12·59 cc. *N*/10 acid. N = 8·7 %.

*Sodium Chloride.* Glycine ester hydrochloride was boiled in aqueous solution with one molecular proportion of anhydrous sodium carbonate. On concentration and allowing the solution to cool slowly or rapidly a mixture of crystals was invariably obtained, the glycine and sodium chloride separating side by side.

*Glycine and Potassium Halides.*

Using molecular proportions of glycine and one of the halides of potassium, namely chloride, bromide or iodide, no evidence was obtained of the formation of a crystalline addition compound. In one experiment with potassium bromide, a homogeneous crop of crystals was obtained. These contained a trace of halide, melted from 227–240°, and when analysed for nitrogen by the Kjeldahl process, proved to be practically pure glycine.

0·2026 g.; $NH_3$ equivalent to 26·58 cc. *N*/10 acid. N = 18·4 %.
Calculated for $C_2H_5O_2N$. N = 18·7 %.

*Other Crystalline Forms of Glycine.*

Glycine usually crystallises in compact crystals in the monoclinic system. When recrystallised from water, we have repeatedly observed the formation of another crystalline form of glycine, separating in long bold needles or columns and devoid of water of crystallisation. In one instance these were about two inches long. On keeping in contact with the solution, they invariably pass into the monoclinic variety, the unstable form gradually dissolving. The purest stable glycine that we have prepared decomposes in the neighbourhood of 256° (uncorr.). This temperature depends to a large extent on the rate of heating. There is also progressive darkening from about 240° onwards. The comparative behaviour of successively crystallised glycine is shown by the following table.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Crude glycine, | becomes pale brown, | about | 220°, | decomposes | 240°. |
| Once crystallised | „ | „ | „ | „ | 225°, | „ | 242°. |
| Twice | „ | „ | „ | „ | 228°, | „ | 247°. |
| Thrice | „ | „ | „ | „ | 235°, | „ | 254°. |
| Four times | „ | „ | „ | „ | 240°, | „ | 256°. |

Fischer's reactive glycine, precipitated by alcohol, prepared from the four times crystallised glycine, when compared side by side in the same bath with the stable form showed an identical behaviour. The observations of Falk and Sugiura ascribing a difference of range of progressive deepening of colour of the two forms on heating, the glycine precipitated by alcohol darkening over a shorter range by 10 to 20°, must therefore be due to the presence of extraneous impurity such as is present in the fewer times crystallised glycine shown above. Moreover, we had available a sample of glycine precipitated by alcohol, from some of which glycine acid chloride had been prepared by Fischer's method. When compared with our purest four times crystallised glycine it became progressively coloured several degrees in advance of the stable form and decomposed at 252°, whereas the stable form, heated in the same bath simultaneously, decomposed at 253°. This again is exactly the contrary of the behaviour described by Falk and Sugiura.

The unstable form from aqueous solution, which was prepared from a sample of pure glycine, when compared with the four times crystallised ordinary glycine, exhibited an identical behaviour.

SUMMARY.

1. The existence of the following addition compounds of glycine prepared by Pfeiffer and his co-workers is confirmed under severer experimental conditions. $BaCl_2, (C_2H_5O_2N)_2, H_2O$; $CaCl_2, (C_2H_5O_2N)_2, 4H_2O$; $SrCl_2, (C_2H_5O_2N)_2, H_2O$; LiCl, $C_2H_5O_2N, H_2O$; LiBr, $(C_2H_5O_2N)_2, H_2O$; LiBr, $C_2H_5O_2N, H_2O$.

2. The following new addition compounds are described. $CaI_2, (C_2H_5O_2N)_2, 3H_2O$; NaBr, $(C_2H_5O_2N)_2, H_2O$; and NaI, $(C_2H_5O_2N)_2, H_2O$.

3. In agreement with Pfeiffer no compounds were obtained with the potassium halides or with sodium chloride.

4. An unstable form of glycine from aqueous solutions is described and its relationships discussed.

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# LI. NOTE ON THE PRODUCTION OF A CONTRACTING CLOT IN A GEL OF GELATIN AT THE ISO-ELECTRIC POINT.

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*Report to the Medical Research Committee.*

*From the Biochemical Laboratory, Cambridge.*

(*Received June 2nd, 1920.*)

With Plate XI.

In a recent paper published in this *Journal* [Jordan Lloyd, 1920] the theory was developed that gelatin gels consist of a solid framework of pure uncombined gelatin, with an interstitial fluid which consists of a solution of gelatin salts, either acidic or basic. Equilibrium in the system was held to be the resultant of two opposing sets of forces, the elastic forces of the solid framework which tend to make the gel contract, and the osmotic forces due to the dissolved salts in the interstitial fluid which tend to keep it expanded. The special case of a hot solution of pure gelatin cooling at a reaction of $P_H = 4{\cdot}6$ was considered, and the following deduction drawn from the theory:

"The iso-electric gelatin will be precipitated at numerous crystallisation centres, the solid drops will run together to form a framework, but since there can be no osmotic forces in the system...the framework will contract under the action of its own surface forces and the internal phase will be squeezed out." This condition of the formation of a contracting clot in a gelatin gel has now been realised in the laboratory and is shown in Plate XI, fig. 1. The gel was prepared by the following method: sheets of Coignet's Gold Label gelatin were dialysed against two changes of $N/1000$ hydrochloric acid, then against three changes of distilled water, and finally against dilute hydrochloric acid at $P_H = 4{\cdot}6$. The dialysing acid was tested after twenty-four hours for change of reaction and was replaced daily by fresh acid at $P_H = 4{\cdot}6$ until no change of reaction after twenty-four hours' dialysis could be detected by the colorimetric method. The indicator used was methyl red, and the colour was standardised by comparison with that obtained by adding an equivalent amount of indicator to a solution consisting of 50 cc. $0{\cdot}2M$ acid potassium phthalate + 13 cc. $0{\cdot}2M$ caustic soda in a total volume of 200 cc. [Clark and Lubs, 1917]. The whole course of dialysis lasted about a month.

The soaked sheets of gelatin were then removed from the dialysing fluid, dried, melted in an evaporating basin and filtered hot into a large volume of 97 % alcohol. A litre of alcohol was allowed to every 100 cc. of melted gelatin. The gelatin was precipitated as a white fibrous mass possessing marked elasticity. It was allowed to stand in the alcohol twenty-four hours,

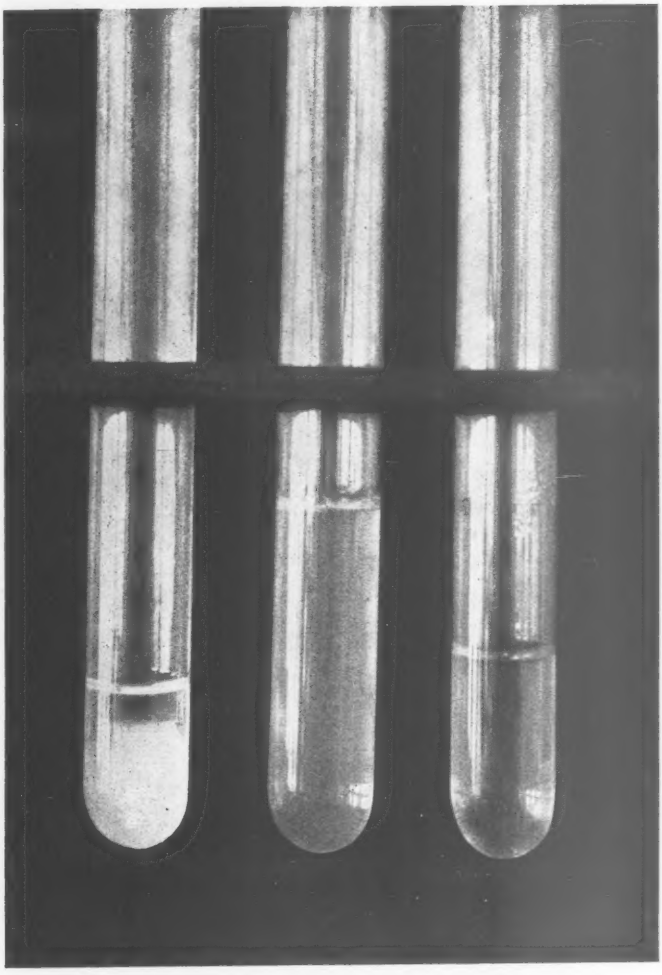

Fig. 1 Fig. 2 Fig. 3

then in three changes of strong alcohol, each for twenty-four hours, and then in three changes of absolute alcohol. Finally the white fibrous mass was washed with ether and dried in a desiccator. This method of preparing pure gelatin differs from that employed by Dheré [1913] and by Dheré and Gorgolewski [1910] in the reaction of the dialysing fluid, but is otherwise similar to that described by these workers. The pure gelatin is snow-white and quite friable. The ash content was 0·1 %. A full account of its properties will be given in a subsequent paper. The three gels made from this gelatin and shown in Plate XI are all of a 1 % concentration. They differ in their content of acid, hydrochloric acid being present in tubes 2 and 3.

*Tube* 1 (fig. 1). The gelatin was dissolved in distilled water by heating, and cooled to 18°. The reaction of the solution at this temperature was $P_H = 6{\cdot}09$. The solution set on standing to an opaque white gel. The contraction of the opaque gel was visible in twenty-four hours. The photograph reproduced was taken after standing for fourteen days. The reaction of the expressed clear fluid was taken again and was found to be $P_H = 4{\cdot}42$. The fluid gave a faint positive biuret reaction.

A portion of the clot was stained with methylene blue and examined under a $\frac{1}{12}''$ oil immersion lens, with compensating eye-piece × 12.

The crushed gelatin clot appeared to consist of numerous small spheres similar to the spherites described by Bradford [1920]. They were however very uniform in size, being about 0·5 $\mu$ in diameter. No formation of filaments was detected. This, however, may have been due to the crushing of the clot under the cover-glass.

*Tube* 2 (fig. 2). The system contained 1 % of gelatin and HCl to a concentration of 0·005 *N*. The reaction of the freshly prepared solution at 18° was $P_H = 3{\cdot}43$. The solution set after two hours to a clear colourless gel, which became faintly opalescent after forty-eight hours' standing. No further change occurred during the following fortnight, *i.e.* till the end of the experiment.

*Tube* 3 (fig. 3). The system consisted of 1 % of gelatin in 0·01 *N* HCl. The reaction of the freshly prepared solution after cooling to 18° was $P_H = 2{\cdot}53$. The solution set to a glassy clear gel which showed no changes in transparency on standing.

These observations demonstrate that gelatin gels are unstable at the iso-electric point. Small quantities of caustic soda added to the system have the same stabilising influence as small quantities of hydrochloric acid. The formation of stable gels therefore is only possible in the presence of electrolytes.

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# LII. AN EXPERIMENTAL STUDY OF THE EFFECT OF CERTAIN ORGANIC AND INORGANIC SUBSTANCES ON THE BREADMAKING PROPERTIES OF FLOUR AND ON THE FERMENTATION OF YEAST.

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(*Received July 2nd, 1920.*)

During the war, when it was necessary to conserve the wheat supply of the country, the proportion of the flour extracted from the grain was greater than in normal times, and other cereals were being milled and added to the wheat.

The loaf made from such flours was small in size and close in texture, and complaints were also made that it showed a tendency to become sour. Although numerous suggestions for improving the breadmaking properties of the flour were forthcoming, the problem thus raised was an extremely complex one.

The factors on which the breadmaking properties of a flour depend are by no means easy to define and exact data on this subject are difficult to obtain.

It was hoped that these investigations of the effect of certain organic and inorganic substances on the breadmaking properties of flour might yield results which would be of some practical assistance in arriving at a satisfactory solution of the problem. At first the method of investigation adopted was that of making baking experiments. Later, in order to check the behaviour of the various ingredients used in breadmaking, and to obtain some indication of the changes which occur during the different stages of the process, measurements were made of the amount of fermentation produced by the yeast in the absence of flour and also of the expansion of the dough whilst "proving" or "rising" prior to baking.

## Baking Experiments.

Loaves were cooked in batches of four, each loaf containing the same weight of the necessary ingredients, *i.e.* flour, raising agent, salt and water, to which might be added different substances which varied with the nature of the experiment.

In order to minimise experimental errors which are almost inevitable in work of this character, one loaf of each batch was prepared as a control and contained the same ingredients in each experiment. In all cases where it was possible, the experiments were repeated two or three times, and an average of the results taken.

*Ingredients of doughs raised with yeast.*

1. 200 grams of flour. The flours used in these experiments were kindly supplied by Messrs J. and R. Robinson, Deptford Bridge Mills, Greenwich, and their composition was as follows:

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| I. | Flour containing | 80 % | of the | wheat | grain. | | |
| II. | „ | „ | 85 % | „ | „ | „ | and barley. |
| III. | „ | „ | 75 % | „ | „ | „ | which was a weak English wheat. |
| IV. | „ | „ | 90 % | „ | „ | „ | and rice, maize, barley and rye. |
| V. | „ | „ | 85 % | „ | „ | „ | and rice, maize, barley and rye. |
| VI. | „ | „ | 75 % | „ | „ | „ | which was a weak English wheat. |
| VII. | „ | „ | 83 % | „ | „ | „ | and barley, rice and rye. |
| VIII. | „ | „ | 85·9 % | „ | „ | „ | and maize, rice and barley. |
| IX. | „ | „ | 85·9 % | „ | „ | „ | and maize, rice and barley. |
| X. | „ | „ | 86 % | „ | „ | „ | and barley, rice and rye. |

In addition some white flour similar to the pre-war flour was obtained by a special permit.

2. 5 grams of yeast supplied by a wholesale firm. The age of the samples was known.

3. 2 grams of sugar (or 10 cc. of a 20 % solution).

4. 2 grams of salt.

5. Liquid for mixing. The volume of liquid required for mixing the dough to the necessary consistency varied from 95–120 cc. according to the nature of the flour. The liquid was chiefly tap water, but the total volume included any other liquid, *e.g.* sugar solution, serum, etc., added for the purpose of the experiment.

*Method of making bread raised with yeast.*

The yeast was mixed to a thin cream with the sugar solution, and added to the flour and salt which had previously been mixed and raised to a temperature of 25°. The rest of the liquid (at 25°) was then added to the dough thus formed and kneaded for five minutes. Longer time could not be allowed as the dough cooled rapidly during this process.

*First rise.* The dough was allowed to rise in an incubator kept at 25° until the surface skin cracked. The time required varied with the nature of the flour. A dough made from a "strong flour" (I) required one hour and that from a "weak flour" (II) forty-five minutes. The addition of cereals other than wheat reduced the time necessary for rising.

The dough was then kneaded and put into a greased tin which had previously been weighed.

The tins were cylindrical so that the approximate volume of the loaf could be readily calculated. The dough was weighed in the tins. This weight served as a check on the manipulation of the mixing.

*Second rise.* The dough was put back into the incubator and allowed to remain there until it had about doubled its size. The time necessary for this second rise varied with the flour in the same manner as for the first rise.

*Baking of the dough.* The dough was put into a hot oven, the temperature raised for the first ten minutes and then decreased at intervals. At first a gas oven was used, but later an electric oven was substituted so that the temperature could be more easily controlled. In some of the later work the second rise was omitted, *i.e.* the dough was mixed, put into the tin, allowed to rise for the necessary time and baked. The same range of results was obtained by this latter method as by the longer process, and any possible error introduced by further handling of the dough was eliminated. Some bakings involving the longer process were carried out in all cases in order to check the results obtained by the shorter method.

*Weight and volume of the loaf.* When cold the loaf was weighed, and after keeping twenty-four hours, it was cut through the centre and the greatest and least heights measured.

The volume of the loaf was calculated from the mean height and the area of the base.

The volume of the control was taken as 1000, and the volumes of the other loaves calculated relatively to this. By this means a comparison between experiments carried out at different times could be obtained.

*Doughs raised by a chemical raising agent.*

The ingredients of these doughs were as follows:

1. 200 grams of flour.
2. 1 gram of sodium bicarbonate and 2 grams of potassium bitartrate.
3. 2 grams of salt.
4. Sufficient liquid to make a dough.

The raising agent was mixed with the flour and salt, and the mixture passed through a sieve. After adding the liquid the dough was kneaded, placed in a weighed tin, weighed and baked as described above.

*The addition of lime-water in breadmaking.*

Bread made from "Government controlled" flour showed a tendency to become sour, which was not observed in the case of pre-war white flour. Jago [1911] states that bread made with lime-water is quite free from sourness.

In order to investigate the power of lime-water to correct the tendency to sourness, baking experiments were carried out with doughs mixed with lime-water, some being raised with yeast and others with a chemical raising agent.

Experiments were made with whole-meal flour, Government controlled flour and white flour.

The loaves made with lime-water were in all cases smaller than their controls. The doughs mixed with lime-water appeared drier than the controls, and whilst rising formed a thick skin.

Some determinations were made of the acidity and also of the moisture content of the lime-water loaves and of their controls, determinations being made both on the freshly baked bread and on bread which had been kept for several days.

The results showed that whilst the lime-water neutralised any acidity in the flour it did not prevent the production of acid.

There was no appreciable difference in the rate of loss of moisture on keeping between the lime-water loaves and the controls. The acidity of the bread decreased as it became drier.

Bywaters [1918] states that the use of lime-water has been largely discontinued as the action of the yeast is thereby delayed. Lapigne and Legendre [1917] and also Le Roy [1917] make reference to this subject.

Observations based on practical experience show that bread which is made on a large scale is more liable to become sour than bread which is made in smaller quantities.

This difference it was thought might be due to the fact that when bread is made on a large scale a longer time is usually allowed for the fermentation or "proving" process than is allowed in a small scale baking.

Some determinations which were made of the acidity of the dough during fermentation showed that the acidity increased slightly as fermentation proceeded. In a typical case the acidity expressed as lactic acid was 0·46 % on the freshly prepared dough and increased to 0·66 % after five hours. This shows that the acidity of the dough is dependent to some extent on the length of time allowed for proving.

It thus appears there is little to be gained by the use of lime-water in breadmaking except, perhaps, in the case of a very acid flour or if the process of fermentation is prolonged.

*Experiments on the physical properties of gluten.*

A study of the technology of breadmaking indicates that the size of loaf is partially dependent on the physical properties of the gluten and that to produce a good loaf the gluten must be moderately elastic.

Experiments were made to see if the elasticity of the gluten could be increased and a larger loaf obtained by stretching the dough by some physical process prior to the addition of the yeast. The dough was placed in a vacuum desiccator and the pressure alternately reduced and then increased again to atmospheric pressure three times.

Some expansion of the dough was produced by this process, but after baking the loaf was not appreciably larger than the control.

### The addition of ox-serum in breadmaking.

At the suggestion of Professor Leonard Hill some experiments were made in which a proportion of ox-serum was added to the liquid used in mixing the dough.

The ox-serum used was separated at the Metropolitan Cattle Market, Islington, and obtained forty-eight hours after the animal had been slaughtered. In cold weather it was possible to keep the serum for three days before it became unfit for use, and experiments were carried out with the serum on the first, second and third day.

Baking experiments were made to ascertain the effect of the serum on the size of the loaf and to find how its action varied with the amount of serum added and the length of time the sample had been kept. The serum was used in proportions varying from 1 to 7 %, expressed by weight in relation to the flour. The results obtained on the addition of fresh serum (first day) in concentrations of 1–3 % with four different flours are shown in Table I.

Table I.

| Concentration of serum | Flour | Weight of loaf in g. | Height of loaf in cm. | | | Volume of loaf | |
|---|---|---|---|---|---|---|---|
| | | | Maximum | Minimum | Mean | Calculated | Comparative |
| First day 1 % | I | 281·0 | 11·25 | 7·25 | 9·25 | 726·8 | 1156 |
| „ „ 2 % | | 282·5 | 11·25 | 7·0 | 9·12 | 716·9 | 1124 |
| „ „ 3 % | | 279·5 | 10·25 | 6·25 | 8·25 | 643·2 | 1023 |
| Control | | 280·9 | 9·75 | 6·25 | 8·0 | 628·6 | 1000 |
| First day 1 % | II | 269·9 | 9·5 | 7·0 | 8·25 | 648·2 | 1048 |
| „ „ 3 % | | 274·9 | 9·75 | 6·25 | 8·0 | 628·6 | 1016 |
| Control | | 274·9 | 9·5 | 6·25 | 7·87 | 618·7 | 1000 |
| First day 1 % | IV | 290·6 | 9·0 | 6·5 | 7·75 | 608·9 | 1051 |
| „ „ 2 % | | 287·6 | 9·0 | 6·5 | 7·75 | 608·9 | 1051 |
| „ „ 3 % | | 292·4 | 8·75 | 6·5 | 7·62 | 599·1 | 1034 |
| Control | | 290·2 | 8·5 | 6·25 | 7·37 | 579·4 | 1000 |
| First day 1 % | III | 251·0 | 8·5 | 6·5 | 7·75 | 589·3 | 1053 |
| Control | | 246·0 | 8·25 | 6·0 | 7·12 | 559·8 | 1000 |

A marked increase in the volume of the loaf resulted from the addition of 1 % fresh serum.

With concentrations greater than 1 %, the increase on the whole was less marked, although with flour IV the same result was obtained with 2 % as with 1 % of serum. The increase obtained varied with the flour; the maximum increase being 15·6 % with flour I.

It should be noted that the addition of the serum affects only the volume, not the weight of the loaf; the slight differences in weight observed are to be attributed to experimental errors.

Further experiments showed that the increase obtained with 1 % of serum was greater than that obtained with concentrations less than 1 % and also that on the addition of 1 % serum to pre-war white flour a decrease of 1·5 % in the volume of the loaf was obtained.

When the serum was used on the second and third days, the loaf obtained with the addition of 1 % serum was considerably smaller than on the first day. The size of the loaf increased with the proportion of serum used, but even when 7 % of serum was added the increase in volume was not so great as that obtained with the addition of 1 % of serum on the first day.

The maximum increase on the different days obtained with flour I were as follows:

| | | | | | | |
|---|---|---|---|---|---|---|
| 1st day | an increase of | 15·6 % | with | 1 % | of serum | |
| 2nd | „ | „ | 12·5 % | „ | 7 % | „ |
| 3rd | „ | „ | 12·5 % | „ | 7 % | „ |

It thus appears that the active principle in serum, which produces an increase in the size of the loaf, is destroyed gradually on keeping, and although the effect of this destruction could to some extent be rectified by increasing the amount of serum used, the maximum effect obtained with the fresh serum could not be produced on subsequent days.

*The effect of heat and preservatives on the properties of serum.*

Some experiments made with serum which had been kept at a temperature of 50–60° for an hour and a half, showed that this treatment had practically no effect on the action of the serum.

Samples of serum, which were heated on arrival and used for baking experiments on the following days, gave a typical first day action on the second day, *i.e.* the maximum increase was obtained with the addition of 1 % of serum, but the increase in the volume of the loaf was not so great as on the first day. Not until the fourth day did the serum heated on the first day give a typical second day reaction, *i.e.* the volume of the loaf increased with the concentration of the serum. Serum heated in this manner did not keep appreciably longer than raw serum. Thus the active principle in the serum is not destroyed by being heated to 50–60°, but it is to some extent protected although not preserved by this treatment.

Serum heated to 100° coagulated and on standing a pale coloured liquid separated out which could be expressed from the solid mass. Baking experiments made with this boiled serum showed that it had very little effect in increasing the volume of the loaf and in some cases the loaf obtained was smaller than the control. The liquid portion was more effective than the solid, but in neither case was a marked increase in volume obtained.

Since the power of the serum to produce an increase in the size of the loaf deteriorates rapidly on keeping, and the maximum effect can only be obtained with fresh serum, some experiments were made to see if it were possible to preserve the serum either by some process of desiccation at low temperatures, or by the addition of chemical preservatives.

An attempt was made to evaporate the serum under reduced pressure at the room temperature, but the process was found to be too slow to be of any practical use.

The protein material in the serum was precipitated by the addition of a saturated solution of ammonium sulphate. The precipitate was filtered and then dried in a vacuum desiccator at the room temperature.

This dried preparation when added to the flour gave similar results to the fresh serum, and did not deteriorate on keeping for a considerable time. Unfortunately the serum obtainable at this stage of the investigations was poor in quality and results yielded were not comparable with those obtained earlier.

The addition of small quantities of formaldehyde (1 part of 40 % formaldehyde to 1000–2000 parts of serum) preserved the serum for several days and in baking on a small scale gave a loaf as large as that obtained with fresh serum; but in one or two experiments which were made on a large scale (see baking on a large scale) a slight decrease was observed.

This may be due to some inhibitory action of the formaldehyde on the yeast owing to the longer time allowed for rising on the large scale, but further experimental work is needed before any definite statement on this point can be made.

Boric acid was found to be ineffective in preserving serum.

*Possible substitutes for serum.*

Defribrinated blood was tried as a substitute for serum; its action was found to be similar to that of second day serum but the loaf was dark in colour.

Solutions of the following substances were also used: gelatin, egg and blood albumin, caseinogen in lime-water.

With caseinogen in lime-water the loaf obtained was smaller than the control. (Comp. the addition of lime-water.)

Gelatin gave an increase of 10 % with flour I, but with the other flours the increase was only from 1–2 %.

A slight increase only (1–2 %) was obtained with albumin and it was not considered that any of these substances could be regarded as a satisfactory substitute for serum.

*The addition of serum to bread raised with a chemical raising agent.*

Serum appeared to have no action on doughs raised with a chemical raising agent; the loaves obtained were the same size as the controls.

*Baking on a large scale.*

In order to decide whether the action of the serum would be the same if the bread were made in larger quantities, one or two experiments were made in which doughs were prepared with 10 lb. of flour, which, after mixing, were divided into eight loaves.

The relative increase in the size of the loaf was less than in the small scale bakings, but the serum was at this time very poor in quality and the experiments were not continued.

Further work is needed to confirm these results and to find whether the optimum concentration of the serum is the same on the large as on the small scale.

The action of serum in the presence of phosphate and of potato is dealt with later.

### The addition of phosphates in breadmaking.

Jago [1911] states that the addition of mineral phosphates to the flour results in a marked improvement of the loaf, but that the addition of such salts is illegal.

Harden [1914] has shown that the addition of phosphates increases the total fermentation produced by a given volume of yeast juice.

Baking experiments were made using monosodium dihydrogen phosphate, $NaH_2PO_4.H_2O$, and monohydrogen disodium phosphate, $Na_2HPO_4.12H_2O$, and also acid calcium phosphate, $CaH_4(PO_4)_2$.

The quantities of phosphate used varied from 0·25–3·0 % of the flour.

The average increase in the volume of the loaf obtained on the addition of 1 % of each of these salts to flour was as follows:

| | |
|---|---|
| Monosodium hydrogen phosphate | +3·5 % |
| Disodium " " | +2·6 % |
| Acid calcium phosphate | −0·75 % |

Although the calcium salt produced a slight decrease in the size of the loaf, it gave a bread which was whiter in colour than the control or that containing the other phosphates. Further experiments showed that the maximum increase in the size of the loaf was obtained on the addition of 0·5 % of monosodium hydrogen phosphate, and that its effect was more pronounced with "weak" than with "strong" flours.

Thus an increase of 7·4 % was obtained with flour III and an increase of only 1·6 % with flour IV.

With disodium hydrogen phosphate the size of the loaf increased with increasing concentrations of the salt up to 3 %, and it was not considered advisable to exceed this amount. The maximum increase obtained with the latter salt was less than that obtained with the monosodium salt.

Judging by the experimental results obtained, there appeared to be no advantage in using a mixture of the salts in the proportion of five molecules of the disodium salt to one of the monosodium salt, although such a mixture was found by Harden [1914] to produce the maximum effect on the fermentation of yeast juice, or in using a mixture of the two salts in the proportions of the concentrations found to produce the maximum effect, *i.e.* 0·5 % of monosodium hydrogen phosphate, and 3 % of disodium hydrogen phosphate.

#### *The addition of phosphates to doughs raised with a chemical raising agent.*

The addition of phosphates to doughs raised with a chemical raising agent did not produce any increase in the size of the loaf.

*The addition of phosphate and serum in breadmaking.*

A comparison of the action of phosphate with that of serum showed that in the case of a weak flour a greater increase in the size of the loaf was obtained by the addition of phosphate than by the addition of serum.

With flour III the addition of 0·5 % of monosodium dihydrogen phosphate produced an increase in the size of the loaf of 7·4 %, and the addition of 1 % of fresh serum an increase of 5·3 %, but with some of the "stronger flours," *e.g.* V and VI, the results were in the reverse order.

If both phosphate and serum were added the increase in the volume of the loaf was greater than that obtained with phosphate alone, but the relative increase in the size of the loaf as compared with serum alone depended on the nature of the flour, *i.e.* the serum and phosphate in most cases produced a larger loaf than the serum alone, but in some cases a smaller one.

## The addition of potato in breadmaking.

In order to economise wheat flour and other cereals, the addition of potato to the flour used for breadmaking has been extensively advocated.

As the production of potato flour involves special plant for drying the potatoes, it was thought that it would be more convenient, providing that equally good results could be obtained, to use either raw or cooked potato instead of potato flour.

The potato was substituted for a portion of the flour, due allowance being made with raw and cooked potato for the water-content of the material, *e.g.* 70 g. of raw potato were taken as equivalent to 19 g. of flour and a mixture of 181 g. of wheat flour and 70 g. of raw potato as equivalent to the 200 g. of flour used in the other baking experiments.

The average results obtained were as follows:

| | |
|---|---|
| Raw potato, increase in the volume of the loaf | 1·2 % |
| Boiled " " " " " " | 4·2 % |
| Potato flour " " " " " " | 1·25 % |

Steamed potato had the same effect as boiled potato.

Baking experiments were also made with potato which had been boiled twice as it has been suggested that all enzymes are destroyed by this treatment. This potato had the same effect as ordinary boiled potato.

Experiments made to determine the best proportions of potato to use showed that the size of the loaf decreased with increasing proportions of raw potato, but increased with increasing proportions of boiled potato.

Thus when the potato and flour were in the proportion of 1 : 2 the increase in the volume of the loaf was 3·6 % with raw potato and 10·7 % with boiled potato, and when the potato and flour were in the proportion of 2 : 1 the increase was 1·8 % with raw potato and 14·5 % with boiled potato.

In the latter case, however, the mixture was quite unsuitable for breadmaking as the upper part of the cooked dough was hollow and the lower part

had the appearance and consistency of cooked batter. On the average the best results were obtained with a mixture of wheat flour and potato, containing boiled potato equivalent to from 25–33 % of the total weight.

The volume of the loaf decreased with increasing proportions of potato flour, but the flour being dry it was possible by this means to introduce a larger proportion of potato and still obtain a loaf which was satisfactory as regards texture and appearance, although smaller than the control. This bread, however, became stale and dry very rapidly. It was observed that the addition of potato made the dough less spongy. Owing to its high water-content raw potato, which was grated before adding to the dough, tended to make the dough moist and considerably less water was required for mixing than was required by the control. The doughs containing raw potato were greyish in colour owing to changes due to enzyme action which take place when raw potato is exposed to the air and this made the bread a bad colour.

In mixing doughs containing boiled potato the amount of water used was in most cases the same as for the control.

*The addition of potato and serum in breadmaking.*

Baking experiments made with the addition of serum to bread containing cooked potato showed that the maximum effect was produced, as in the case of flour alone with the addition of 1 % of fresh serum.

The increase in volume of the loaf obtained with fresh serum in the presence of potato was slightly less than when the potato was omitted, the average difference in the results being about 1 %. Further, when the serum was used on the second day the addition of 2 % of serum gave the maximum increase and the same result was obtained on the third day.

During a spell of cold weather it was possible to bake with one sample of serum on the fourth day. The volume of loaves containing potato and serum then increased with the amount of serum added, *i.e.* the action of the serum was then the same as that observed with second day serum when flour alone was used.

*The addition of potato and malt extract in breadmaking.*

The addition of malt extract to dough containing potato did not increase the size of the loaf, and if the proportion of potato to flour was more than 1 : 2 the loaf became smaller on the addition of malt.

It was previously noted that with boiled potato a larger loaf was obtained when the proportion of potato was increased, but on the addition of malt and potato the size of the loaf decreased as the proportion of potato increased.

Further, it was found that the amount of boiled potato which could be introduced into the bread was less in the presence of malt, as the bread showed a tendency to become extremely heavy.

## Experiments on the fermentation of yeast and measurements of the expansion of the dough during rising.

In order to check, as far as possible, the behaviour of the various substances used in making the bread, and to obtain some indication of the changes which occur at the different stages of the process, measurements were made of the amount of fermentation produced by the yeast with the various ingredients in the absence of flour and also of the expansion of the dough prior to baking.

### *Experiments on the fermentation of yeast.*

The fermentation experiments were carried out in 100 cc. flasks, each flask being attached to a nitrometer, filled with brine, in which the carbon dioxide evolved could be collected and measured. The flasks were placed in a thermostat kept at a temperature of 25°.

The weights of yeast, sugar, and any additional substance were exactly one-tenth of the weights used in the baking experiments, and, except where otherwise stated, the total volume of liquid was 10 cc.

The experiments were carried out in batches of four. One of each batch contained yeast, sugar, and water only, and was used as a control, and each experiment was done in duplicate.

The mixture to be fermented was placed in a flask and the volume of carbon dioxide evolved read off at regular intervals.

### *Experiments on the expansion of dough.*

The proportions of flour, yeast, etc., used in these experiments were exactly half those used in the baking experiments; it was thus possible to get a direct comparison between the baking experiments, the expansion of the dough, and the fermentation experiments.

The dough was introduced into wide-necked glass bottles fitted with rubber bungs through which delivery tubes were passed. The volume of the air displaced by the rising of the dough was measured by the displacement of water. The water was kept alkaline by the addition of sodium hydroxide, so that any carbon dioxide which escaped through the dough should be absorbed.

It was felt that the experiments would be more complete if some arrangement could be devised by which the expansion of the dough during baking as well as during the fermentation process could be measured.

An apparatus for this purpose was constructed of tinned copper, but although several different types of washer have been tried it has so far not been possible to make the screw top absolutely air-tight at the baking temperature, and reliable results have not yet been obtained.

*Results of experiments on the fermentation of the yeast and the expansion of the dough.*

Experiments were first made to compare the amount of carbon dioxide produced by the yeast, sugar, and water only with the expansion of the dough during fermentation.

The carbon dioxide evolved by the fermentation of the yeast was measured at the end of one hour and at the end of four hours.

It was found that the volume of the gas produced varied with the age of the yeast, and the maximum amount of fermentation was produced, not with fresh yeast, but with yeast that was two to three days old.

The amount of fermentation produced varied considerably with the different samples of yeast; the worst sample produced 2·3 cc. during the first hour and a good sample 18·4 cc. in the same time.

These results were confirmed by measurements of the expansion of the dough (see Table II).

Table II.

*Expansion of the dough during fermentation.*

| Age of yeast. Days | Increase in volume of the dough. 20 mins. cc. | 40 mins. cc. | 60 mins. cc. | 80 mins. cc. |
|---|---|---|---|---|
| 1 | 10·0 | 52·0 | 121 | — |
| 2 | — | 62·0 | 136·6 | 230 |
| 3 | 15·0 | 61·5 | 124 | 202 |
| 4 | 12·0 | 54·0 | 123 | 210 |

Fermentation experiments, measurements of the expansion of the dough and baking experiments were carried out on the same day with the same sample of yeast.

The fermentation produced by the yeast and the expansion of the dough were measured as described above, and the results which would be obtained with the quantities used in the baking experiments were then calculated. A dough was also prepared and baked without the addition of the yeast to act as a control, and it was assumed that the volume of the loaf less the volume of this cooked dough (422·3 cc., see Table III) and less the expansion of dough during fermentation, might be taken as a measure of the expansion on baking.

Thus in Table III the figures in column VI are obtained by subtracting the volume of the baked control (422·3 cc.) and the expansion of the dough (column III) from the volume of the loaf (column V).

As might be expected the amount of fermentation produced in the dough is greater than with the yeast alone, this probably being due to the presence of substances in the flour, *e.g.* gluten, etc., which act as yeast foods.

Further there is a maximum increase on baking which is obtained (under the conditions of the experiment) after the dough has risen for 40 minutes.

Table III.

| I | II | III | IV | V | VI | VII |
|---|---|---|---|---|---|---|
| Age of yeast Days | Time of fermentation Minutes | Fermentation of yeast (5 g.) cc. | Expansion of dough during fermentation (5 g. yeast) cc. | Volume of loaf (5 g. yeast) cc. | Expansion on baking cc. | Ratio of fermentation to expansion of dough (III : IV) |
| — | nil | — | — | 422·3 | — | — |
| 1 | 20 | 6 | 10 | 510·7 | 78·4 | 1 : 1·7 |
| | 40 | 28 | 52 | 579·5 | 105·2 | 1 : 1·9 |
| | 60 | 64 | 121 | 628·6 | 85·3 | 1 : 1·9 |
| 2 | nil | — | — | 422·3 | — | — |
| | 40 | 31 | 62 | 599·1 | 114·8 | 1 : 2 |
| | 60 | 72 | 136 | 628·6 | 70·3 | 1 : 1·9 |
| | 80 | 115 | 230 | 658·1 | 5·8 | 1 : 2 |
| 3 | 20 | 8 | 15 | 500·9 | 63·6 | 1 : 1·9 |
| | 40 | 31 | 61 | 569·1 | 85·8 | 1 : 1·9 |
| | 60 | 71 | 124 | 617·1 | 70·8 | 1 : 1·8 |
| 4 | 20 | 6 | 12 | 520·5 | 86·2 | 1 : 2 |
| | 40 | 31 | 54 | 579·5 | 103·2 | 1 : 1·7 |
| | 60 | 68 | 123 | 628·6 | 83·3 | 1 : 1·8 |
| | 80 | 110 | 210 | 658·0 | 25·7 | 1 : 1·9 |

These results show that the ratio of the fermentation of the yeast to the expansion of the dough is fairly constant.

This fact may be of practical significance, for if the maximum increase on baking could be ensured the loaf would be increased in volume by a physical process instead of by further fermentation which utilises and wastes some of the ingredients of the dough.

*The action of serum and albumin during fermentation.*

At the time when these fermentation experiments were made, satisfactory samples of fresh serum were not obtainable, but some measurements were made of the fermentation produced by yeast and the expansion of the dough in the presence of serum which had been "salted out" of solution and dried in the manner previously described. This serum contained a small amount of ammonium sulphate, but control experiments made with this salt showed that in small quantities it was without action on the yeast.

The serum used had been kept for twelve months. The addition of this serum increased the volume of carbon dioxide produced during fermentation and also the amount of expansion in the dough.

Thus the increase in volume of the loaf observed on the addition of serum must to some extent at least be attributed to the action of the serum as a yeast stimulant.

Some measurements made with albumin showed that it had some stimulating action on the yeast, but that this action was less marked in the dough.

*The action of phosphates during fermentation.*

Fermentation experiments were made with yeast, sugar and phosphates in the proportions found by the baking experiments to give the best results.

In the first series of experiments the phosphate appeared to exert an inhibitory action on the yeast, as the volume of carbon dioxide evolved was less than from the control.

This it was thought might be due to the fact that although the phosphate and yeast were in the same proportions as in the baking experiments, they were used in a more concentrated form.

Harden [1914] found that when phosphate is added to fermenting yeast juice and sugar, the effect varies with the concentration and that with a mixture of 25 cc. of yeast juice and 4·5 g. of sugar, 5–10 cc. of 0·3 molar solution of phosphate in a total volume of 45 cc. produced the maximum effect.

Fermentation experiments were therefore made using 0·5 g. of yeast, 0·125 g. of cane sugar with from 2 to 40 cc. of 0·3 molar solution of monosodium dihydrogen phosphate in a total volume of 45 cc. Table IV shows the results of a typical experiment.

Table IV.

| Volume of 0·3 molar solution of phosphate cc. | Percentage increase in fermentation of yeast on the addition of phosphate. | | | |
|---|---|---|---|---|
| | 1 hour | 2 hours | 3 hours | 4 hours |
| 2 | 0 | 3·6 | 4·3 | 1·5 |
| 5 | 0 | 14·3 | 14·9 | 11·0 |
| 10 | 8·3 | 17·9 | 17·0 | 13·2 |
| 20 | 33·3 | 44·9 | 40·4 | 29·4 |
| 30 | 41·7 | 50·0 | 44·7 | 35·3 |
| 40 | 41·7 | 46·4 | 40·4 | 29·4 |

These results show that the maximum increase was obtained with 30 cc. of the 0·3 molar solution (= 0·12 g. of $NaH_2PO_4.H_2O$), *i.e.* with 0·12 g. of phosphate and 0·5 g. of yeast in 45 cc., which corresponds to 1·2 g. of phosphate and 5 g. of yeast in 450 cc.

The optimum increase on baking was obtained with 1 g. of phosphate and 5 g. of yeast in about 330 g. of dough, so that the results of fermentation experiments confirm those obtained in the baking experiments.

*The action of potato during fermentation.*

In order to study the effect of the addition of potato on the fermentation of yeast, experiments were made with the following preparations of potato:

I. *Raw Potato*: (*a*) peeled potato, (*b*) peeled potato washed, (*c*) juice expressed from potato, (*d*) potato peel, (*e*) washed potato peel, (*f*) juice extracted from potato peel.

The solid matter was finely grated before use.

II. *Cooked Potato*: (*a*) boiled potato (mashed), (*b*) steamed potato, (*c*) boiled potato (washed), (*d*) potato which had been boiled twice. (These potatoes were boiled for 20 minutes, left over night and boiled again until cooked.)

III. *Potato Flour.*

In the first experiments 4 g. of potato were used with 0·5 g. of yeast, but later it was found that a more marked effect was obtained when the same weight of yeast was used with 10 g. of potato.

Table V.

| Condition of potato | Weight of potato g. | Average percentage increase in the fermentation of yeast with sugar and water produced by addition of potato | | | | |
|---|---|---|---|---|---|---|
| | | 1 hour | 2 hours | 3 hours | 4 hours | 5 hours |
| Raw potato peeled ... ... | 4 | 68·9 | — | — | 31·2 | — |
| „ „ „ and washed | 4 | 48·2 | — | — | 8·9 | — |
| Potato peel ... ... ... | 4 | 78·1 | — | — | 29·7 | — |
| „ „ washed ... ... | 4 | 48·8 | — | — | 12·0 | — |
| Juice of potato ... ... | 4 | −29·4 | — | — | 12·2 | — |
| Juice from potato peel ... | 4 | −59·8 | — | — | −25·8 | — |
| Raw potato ... ... ... | 10 | 92 | 84 | 69 | 64 | 64 |
| Juice of potato ... ... | 10 | −28·7 | 5·5 | 22 | 28 | — |
| Boiled potato ... ... ... | 10 | 82 | 61 | 35 | 21 | 21 |
| Steamed potato ... ... | 10 | 93 | 84 | 60 | 35 | 33 |
| Potato flour ... ... ... | equivalent to 10 g. of potato (*i.e.* 2 g.) | 34 | 25 | 20 | — | — |
| Juice of potato filtered and boiled ... ... ... | 10 | 16 | 36 | 54 | 64 | 77 |
| Juice of potato filtered, boiled and filtered ... ... | 10 | 9 | 24 | 39 | 48 | 58 |

These results show that raw potato produced a marked increase in fermentation, but that if the potato were washed until the liquid filtering through was clear this increase was appreciably diminished.

The juice extracted from the potato inihibited fermentation at first, but later produced a slight increase.

The increase produced by raw potato was greater than the sum of those produced by the washed potato and the juice of the potato.

Potato peel increased the fermentation of the yeast to a greater extent than raw potato. This might be due to the higher water-content of the former, *i.e.* the total solid matter added was greater in the case of the peel.

The juice extracted from the peel had a more marked inhibitory action than the juice from the potato, and the combined effect of the washed peel and the juice of the peel acting separately was less than that of the peel.

Boiled potato did not increase the fermentation to the same extent as raw potato, but steamed potato had the same effect as raw potato for the first two hours and its stimulating power then became less marked.

This difference in the behaviour of boiled and steamed potato is doubtless due to loss of material in the cooking water during boiling and it is possible

that if the potato were boiled in a small volume of water and the cooking water, as well as the potato, used this difference would be eliminated.

The stimulating action of the potato flour was even less than that of washed potato.

When the juice extracted from the potato was filtered and the filtrate boiled, the filtrate became slightly turbid, this being due no doubt to the cooking of minute particles of starch which had passed through the filter. On filtering this boiled liquid a clear green filtrate was obtained.

A comparison of the results obtained with this boiled juice with those obtained with the raw juice indicates that the boiling to some extent destroyed the principle of the raw juice which exerted an inhibitory action on the yeast. Juice which was only filtered once gave a better result than that which was filtered again after boiling.

Observations made on the colour of this boiled juice showed that when grated potato was exposed to the air it became first pink and then rapidly passed through shades of red until it was almost black. When the juice extracted from this potato was boiled and filtered, the filtrate was always green in colour and the depth of colour increased with the length of time the potato was exposed to the air before the juice was extracted.

It has previously been observed that the yeast showed an optimum activity on the second or third day when fermenting with sugar and water, but in the presence of potato it was found that the yeast was more active on the first day.

The results given in Table IV indicate that the increase in fermentation produced by the addition of potato gradually diminished as fermentation proceeded. This effect, which was also observed in the case of other substances which acted as yeast stimulants, may be due to the fact that the apparatus used was such that an equal but limited supply of oxygen was available in each experiment. The substance which stimulated the yeast may thus have been unable to exert its full action towards the end of the experiment owing to the partial exhaustion of the oxygen.

Measurements made of the expansion of dough containing potato showed considerable variations.

Raw potato produced an average decrease in volume of dough as compared with the control of 10·8 %, whilst boiled potato gave a decrease of 5·7 %. The juice from raw potato inhibited the expansion of the dough and washed potato showed the same effect as raw potato but in a greater degree. Steamed potato decreased the expansion of the dough but to a less extent than boiled potato.

The fermentation experiments as a whole form a valuable check on the baking experiments and also demonstrate the necessity of studying the changes involved during the different stages and not the final stage only if any reliable information as to the processes on which breadmaking is based are to be obtained.

Thus, although the fermentation experiments are in general in agreement with the baking experiments this is not always the case. For example, raw potato stimulated the action of yeast to a greater extent than boiled potato, but the loaf obtained with boiled potato was larger than that obtained with raw potato; also the doughs containing potato were smaller than the controls, the difference being less for cooked than for raw potato, but the loaves containing potato were larger than the controls.


## Summary.

The amount of fermentation produced by yeast with sugar and water and in the dough varied with the age of the yeast. The maximum effect was obtained on the second or third day after the yeast had been compressed.

The ratio of the fermentation of the yeast with sugar and water to the fermentation in the dough was approximately constant.

The maximum increase in volume on baking was obtained, under the conditions of the experiments, when the dough was allowed to rise for forty minutes, but this factor may vary with the proportions of the different ingredients used. The acidity of the dough increased with the time of fermentation and the acidity of the bread decreased with the moisture content.

The addition of lime-water to bread does not appear to prevent the production of acid, but it neutralises any acid formed; in the presence of lime-water a smaller loaf was obtained.

On the addition of fresh ox-serum the maximum increase in the size of the loaf was produced with 1 part of serum to 100 parts of flour. The power of the serum to increase the size of the loaf diminished rapidly on keeping, but was not destroyed by heating to a temperature of 50–60°.

With phosphates the best results were obtained when monosodium dihydrogen phosphate was added in the proportion of 1 part of phosphate to 200 parts of flour. Neither phosphate nor serum produced any increase in the size of the loaf when the bread was raised with a chemical raising agent.

Raw potato, cooked potato and potato flour, when substituted for an equivalent amount of wheat flour, diminished the expansion of the dough on fermentation, but both raw and cooked potato gave a larger loaf, the increase being greater with cooked than with raw potato.


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# LIII. AMMONIA EXCRETION, AMINO-ACID EXCRETION AND THE ALKALINE TIDE IN SINGAPORE.

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## Introduction.

In a paper on nitrogen partition in the urine of the races in Singapore [Campbell, 1919] it was noted that on many occasions the amount of ammonia excreted by apparently normal individuals was high when compared with the European standards. It was suggested that the climatic conditions—hot, moist and still air—in Singapore might be responsible for this acidosis.

The observations recorded in the present paper were made in the first place to determine the hourly variations in the ammonia excretion and their significance, which might explain the acidosis. The excretion of amino-acids and the reaction of the urine were also considered.

## Ammonia Excretion.

The amounts of ammonia excreted hourly by thirty subjects of various races, were estimated by Malfatti's method. In most cases daily amounts, for a week or longer, were also estimated using both Malfatti's and Folin's methods. The former method estimates the ammonia of any amino-acids in the urine in addition. Folin's method was not used in my previous paper [1919], but the results obtained with it corresponded with the results recorded by Malfatti's method, as will be seen from Table I, which shows typical examples of average figures from individuals on their usual diets, mixed or vegetarian, similar to those described in the previous paper. In some cases the average figures for ammonia excreted daily were normal; in others, *e.g.* Chinese II and Tamil IV, the figures were high, due allowance being made for the diets and the total nitrogen excretions. Typical examples of all the figures obtained for ammonia excreted hourly are given in Tables II, III and IV. In Charts I, II and III the results, arranged in the form of curves, cover various periods of time continuous for about four days.

On a total nitrogen excretion of about 8 g. the average daily amount of ammonia nitrogen excreted by a normal person is usually stated to be between 0·5 g. and 0·55 g., if Malfatti's method of estimation be used; this gives

Table I. *Ammonia excretion and reaction of urine (average daily figures).*

| Subject | Height in inches | Weight in kilos | Diet | Number of observations | Total *N* g. | Ammonia *N* Folin's method g. | Folin's method % | Malfatti's method g. | Malfatti's method % | Amino-acid *N* g. | Amino-acid *N* % | Total *N* per kilo of body weight | Acidity Leathes' method % | Volume in cc. | Titratable acid in cc. of 0·1 *N* acid *A* | $NH_3$ in cc. of 0·1 *N* acid *N* | Total acidity *A*+*N* | Ratio $\frac{A}{N}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| European | 67·0 | 54·8 | mixed | 16 | 10·54 | 0·55 | 5·3 | 0·68 | 6·5 | 0·13 | 1·2 | 0·192 | — | 1210 | 275 | 396 | 671 | 0·69 |
| Tamil I ... | 69·0 | 54·8 | mixed, mainly rice | 17 | 8·64 | 0·46 | 5·3 | 0·58 | 6·7 | 0·12 | 1·4 | 0·157 | — | 622 | 271 | 328 | 599 | 0·82 |
| Chinese I | 62·5 | 54·8 | ,, | 9 | 8·99 | 0·44 | 4·9 | 0·56 | 6·3 | 0·12 | 1·4 | 0·164 | 55 | 857 | 347 | 319 | 666 | 1·08 |
| Chinese II | 65·0 | 50·0 | ,, | 9 | 7·74 | 0·55 | 7·1 | 0·65 | 8·5 | 0·11 | 1·4 | 0·155 | 59 | 839 | 330 | 392 | 722 | 0·84 |
| Chinese III | 61·5 | 47·4 | ,, | 10 | 9·83 | 0·50 | 5·1 | 0·63 | 6·4 | 0·13 | 1·3 | 0·207 | 51 | 1164 | 345 | 360 | 705 | 0·96 |
| Eurasian... | 61·0 | 51·3 | ,, | 10 | 9·01 | 0·47 | 5·2 | 0·58 | 6·4 | 0·11 | 1·2 | 0·175 | 57 | 1099 | 343 | 339 | 682 | 1·01 |
| Sikh I ... | 65·0 | 52·0 | ,, | 9 | 7·17 | 0·39 | 5·5 | 0·50 | 7·0 | 0·11 | 1·5 | 0·137 | 52 | 1016 | 273 | 279 | 552 | 0·97 |
| Sikh II ... | 61·5 | 44·0 | ,, | 9 | 5·02 | 0·31 | 6·1 | 0·39 | 7·7 | 0·08 | 1·6 | 0·111 | 52 | 511 | 193 | 224 | 417 | 0·86 |
| Tamil II ... | 62·0 | 46·8 | vegetarian, mainly rice | 7 | 4·95 | 0·39 | 7·9 | 0·45 | 9·1 | 0·06 | 1·2 | 0·105 | — | 807 | 162 | 280 | 442 | 0·58 |
| Tamil III | 65·0 | 40·0 | ,, | 17 | 4·57 | 0·29 | 6·3 | 0·35 | 7·7 | 0·06 | 1·4 | 0·114 | — | 895 | 94 | 207 | 301 | 0·45 |
| Tamil IV | 63·5 | 51·1 | ,, | 10 | 5·02 | 0·50 | 10·0 | 0·60 | 12·0 | 0·10 | 1·9 | 0·098 | 39 | 649 | 196 | 357 | 553 | 0·55 |
| Tamil V ... | 65·0 | 52·0 | ,, | 10 | 4·38 | 0·39 | [9·0 | 0·46 | 10·6 | 0·07 | 1·6 | 0·084 | 46 | 699 | 218 | 280 | 498 | 0·78 |
| Standards for Europe | — | — | mixed | — | 16·00 | 0·53 | 3·3 | — | — | — | — | 0·200 | — | 1231 | 278 | 370 (Cole) | 649 | 0·75 |
| | — | — | ,, | — | 8·00 | 0·40 | 5·0 | 0·52 | 6·5 | — | — | — | — | — | — | — | — | — |

Table II.

*Chinese Subjects.* 21/11/19.

(Mixed diet; small meal 6.15 a.m.; large meal 11 a.m. to 12 noon; total nitrogen, 8 g.)

A.—*Urinary Alkalinity per cent. per hour.*

| | a.m. | | | | | | p.m. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average |
| T.S.W. | 36 | 50 | 53 | 34 | 30 | 34 | 44 | 66 | 64 | 34 | 44 |
| L.E.K. | 40 | 55 | 98 | 66 | 50 | 56 | 60 | 70 | 65 | 36 | 59 |
| H.S. | 56 | 71 | 60 | 47 | 37 | 34 | 37 | 42 | 56 | 34 | 47 |
| T.S.H. | 46 | 86 | 97 | 78 | 23 | 23 | 23 | 71 | 94 | 75 | 61 |

B.—*Urinary Ammonia mg. per hour.*

| | a.m. | | | | | | p.m. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average | Remarks |
| T.S.W. | 41 | 38 | 33 | 44 | 29 | 33 | 33 | 20 | 34 | 25 | 33 | Excess |
| L.E.K. | 36 | 28 | 14 | 27 | 19 | 23 | 25 | 17 | 22 | 20 | 23 | Normal |
| H.S. | 25 | 37 | 31 | 34 | 22 | 38 | 40 | 23 | 36 | 28 | 31 | Excess |
| T.S.H. | 22 | 16 | 13 | 16 | 13 | 20 | 20 | 17 | 8 | 13 | 16 | Normal (total nitrogen 5 g.) |

C.—*Amount of Urine cc. per hour.*

| | a.m. | | | | | | p.m. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average |
| T.S.W. | 98 | 172 | 54 | 51 | 35 | 36 | 39 | 31 | 48 | 28 | 59 |
| L.E.K. | 19 | 29 | 30 | 35 | 23 | 42 | 65 | 42 | 42 | 28 | 35 |
| H.S. | 44 | 92 | 100 | 64 | 26 | 24 | 20 | 15 | 28 | 18 | 43 |
| T.S.H. | 46 | 36 | 36 | 34 | 20 | 22 | 22 | 34 | 30 | 25 | 30 |

D.—*Amount of Fluid taken cc.*

| | | Total | Average per hour |
|---|---|---|---|
| T.S.W. | 6.20 a.m., 200 cc.; 9, 200 cc.; 11.20, 200 cc.; 3 p.m., 200 cc. ... | 800 | 80 |
| L.E.K. | 6.20 a.m., 200 cc.; 8.30, 200 cc.; 11, 400 cc.; 3.30 p.m., 200 cc. ... | 1000 | 100 |
| H.S. | 6.15 a.m., 400 cc.; 8.50, 200 cc.; 11, 600 cc. 1 p.m., 200 cc.; 2, 200 cc.; 3, 200 cc. ... ... ... ... ... ... ... ... | 1800 | 180 |
| T.S.H. | 6.30 a.m., 200 cc.; 11.15, 200 cc.; 1.30 p.m., 400 cc.; 3.15, 200 cc. | 1000 | 100 |

the average hourly amount of ammonia nitrogen as 21 or 23 mg. Some of my subjects, whose figures are shown in Tables II, III and IV, excreted quite normal average hourly amounts, whilst others excreted large amounts. The subjects, whose results are given in Charts I, II and III, excreted normal average hourly amounts; and it will be noted that as a rule the ammonia excretion increased as the alkalinity per cent. decreased and *vice versa*; in other words the hourly ammonia varied inversely as the alkalinity or directly as the acidity. This supports the theory that urinary ammonia is there to neutralise acid. The alkalinity per cent. was estimated by Leathes' titration method [1919] in which the reaction of the urine is expressed as so much per cent. alkali or so much per cent. acid.

Attention is now directed to the hourly variations in amount of ammonia excreted when the average was distinctly higher than the normal, that is about 30 mg. As seen in Tables II, III and IV, the hourly variations for large quantities did not differ materially from those for normal amounts, that is the ammonia excreted varied, as a rule, inversely as the alkalinity.

Table III.

*Tamil Subjects.* 24/11/19.

(Mixed diet; small meal 6.15 a.m.; large meal 11 a.m. to 12 noon; total nitrogen, 8 g.)

A.—*Urinary Alkalinity per cent. per hour.*

| | a.m. | | | | | | p.m. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average |
| A.B.C. | 26 | 34 | 62 | 89 | 75 | 53 | 41 | 73 | 66 | 60 | 57 |
| A.P.P. | 45 | 30 | 41 | 40 | 44 | 39 | 36 | 55 | 53 | 52 | 43 |
| V.D.S. | 40 | 41 | 42 | 48 | 50 | 46 | 40 | 62 | 72 | 71 | 51 |
| C.S. | 44 | 56 | 73 | 66 | 47 | 52 | 37 | 60 | 65 | 65 | 56 |

B.—*Urinary Ammonia mg. per hour.*

| | a.m. | | | | | | p.m. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average | Remarks |
| A.B.C. | 68 | 56 | 27 | 33 | 28 | 30 | 42 | 26 | 32 | 31 | 37 | Excess |
| A.P.P. | 21 | 27 | 29 | 33 | 23 | 25 | 25 | 26 | 23 | 28 | 26 | Normal |
| V.D.S. | 33 | 26 | 27 | 40 | 20 | 27 | 23 | 19 | 15 | 17 | 24 | ,, |
| C.S. | 21 | 27 | 19 | 23 | 17 | 26 | 27 | 27 | 12 | 13 | 21 | ,, |

C.—*Amount of Urine cc. per hour.*

| | a.m. | | | | | | p.m. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average |
| A.B.C. | 74 | 144 | 45 | 84 | 90 | 80 | 120 | 43 | 37 | 40 | 75 |
| A.P.P. | 25 | 26 | 43 | 66 | 52 | 50 | 39 | 27 | 29 | 48 | 40 |
| V.D.S. | 100 | 52 | 41 | 60 | 35 | 38 | 24 | 26 | 20 | 25 | 42 |
| C.S. | 32 | 24 | 24 | 26 | 18 | 27 | 31 | 164 | 32 | 53 | 43 |

D.—*Amount of Fluid taken cc.*

| | | Total | Average per hour |
|---|---|---|---|
| A.B.C. | 6.30 a.m., 200 cc.; 11.20, 200 cc.; 2 p.m., 200 cc. ... ... ... | 600 | 60 |
| A.P.P. | 6.30 a.m., 200 cc.; 11.30, 300 cc.; 2 p.m., 200 cc. ... ... ... | 700 | 70 |
| V.D.S. | 6 a.m., 200 cc.; 8, 200 cc.; 11.30, 200 cc.; 2 p.m., 200 cc. ... ... | 800 | 80 |
| C.S. | 6.30 a.m., 200 cc.; 10.30, 200 cc.; 12.15 p.m., 300 cc. ... ... | 700 | 70 |

The main object of the hourly estimations was to endeavour to determine whether the ammonia was excreted in greater quantity during the hours of the day when individuals were most exposed to the effects of the climate. This was not found to be the case. The average hourly ammonia excreted during the period of sleep at night was practically the same as that for the whole day (Charts I, II and III). The total amount for any period of eight consecutive hours of the day was not materially different from that for another period of the same duration. Nevertheless, it must be stated, that

the highest daily amounts of ammonia, in all my cases covering a period of six years, were excreted by those who were, during their work, most exposed to the effects of the climate; they included bakers, rubber estate coolies and rickshaw runners [1918]. In other countries large amounts of ammonia have been detected in the urine of soldiers [Hammarsten and Hedin, 1914]. Koizumi [1919] has found that muscular work at raised temperature causes an increase of urinary ammonia of rabbits.

The subjects described in the present paper attended the medical school, most of them living at the school hostel. Sometimes they showed quite normal amounts of ammonia; at other times there was a distinct increase, the increase lasting sometimes only one day, sometimes several days and sometimes a week, the diet, general routine and total nitrogen excretion remaining almost constant. The average increase for those on a mixed diet

Table IV.

*Eurasian Subjects.* 26/11/19.

(Mixed diet; small meal 6.15 a.m.; large meal 11 a.m. to 12 noon; total nitrogen, 8 g.)

A.—*Urinary Alkalinity per cent. per hour.*

| | a.m. | | | | | | p.m. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average |
| L. | 38 | 44 | 56 | 50 | 50 | 34 | 30 | 41 | 55 | 62 | 46 |
| G. | 36 | 34 | 35 | 35 | 31 | 38 | 30 | 40 | 41 | 42 | 36 |
| N. | 35 | 35 | 46 | 38 | 38 | 38 | 38 | 57 | 87 | 78 | 42 |
| P. | 30 | 40 | 42 | 46 | 51 | 40 | 32 | 54 | 75 | 75 | 48 |

B.—*Urinary Ammonia mg. per hour.*

| | a.m. | | | | | | p.m. | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average | Remarks |
| L. | 45 | 39 | 30 | 49 | 20 | 32 | 32 | 40 | 33 | 34 | 35 | Excess |
| G. | 62 | 31 | 29 | 45 | 25 | 30 | 34 | 32 | 28 | 32 | 35 | „ |
| N. | 25 | 25 | 48 | 36 | 36 | 35 | 35 | 25 | 21 | 21 | 31 | „ |
| P. | 33 | 29 | 25 | 37 | 21 | 19 | 19 | 17 | 26 | 19 | 24 | Normal |

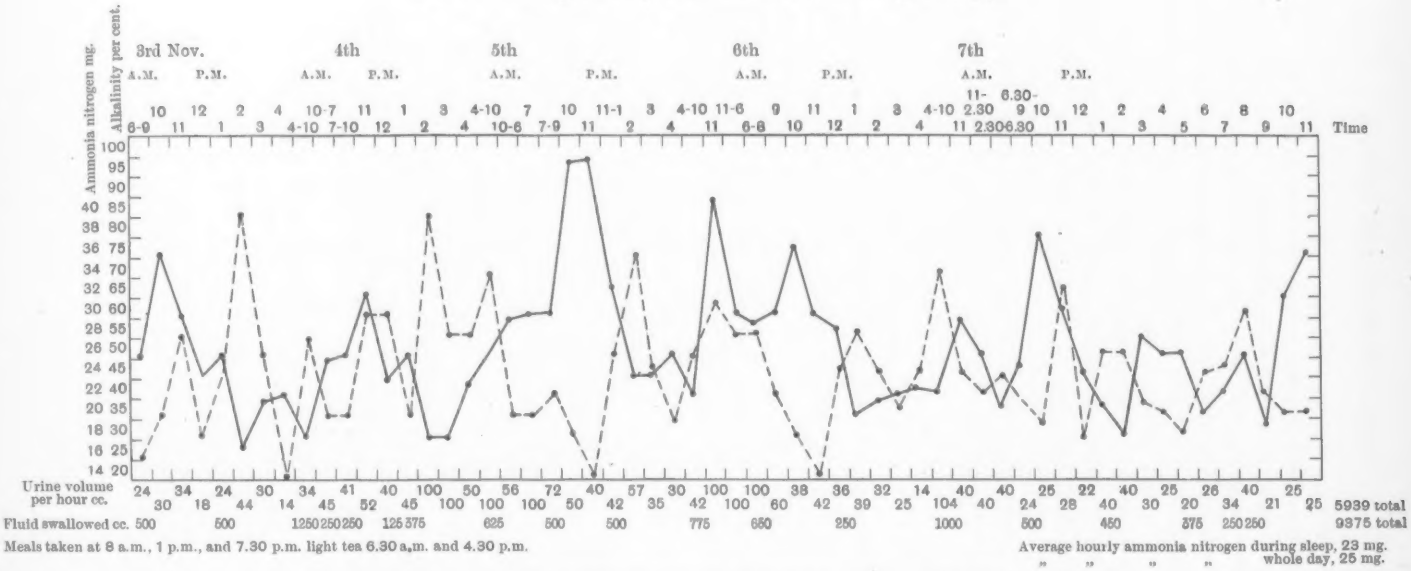
C.—*Amount of Urine cc. per hour.*

| | a.m. | | | | | | p.m. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Subjects | 7 | 8 | 9 | 10 | 11 | 12 | 1 | 2 | 3 | 4 | Average |
| L. | 40 | 38 | 28 | 49 | 20 | 48 | 44 | 49 | 38 | 45 | 39 |
| G. | 68 | 45 | 38 | 80 | 30 | 45 | 55 | 27 | 27 | 30 | 44 |
| N. | 11 | 10 | 54 | 26 | 26 | 40 | 40 | 28 | 44 | 58 | 33 |
| P. | 24 | 22 | 20 | 30 | 18 | 14 | 12 | 20 | 32 | 26 | 21 |

D.—*Amount of Fluid taken cc.*

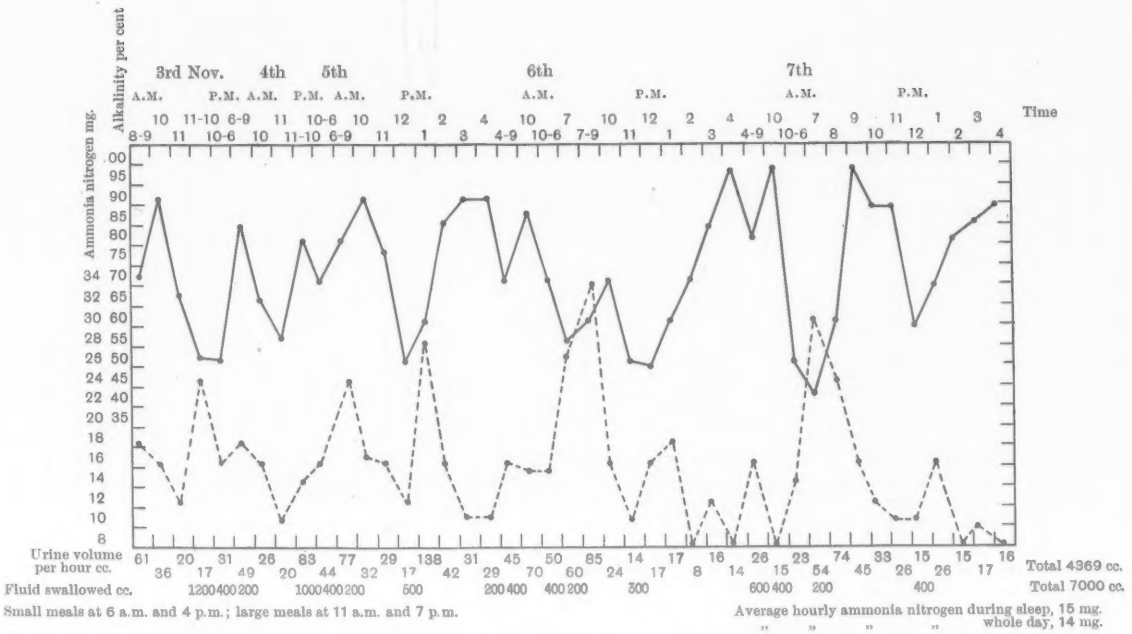
| | | Total | Average per hour |
|---|---|---|---|
| L. | 6.15 a.m., 200 cc.; 8.45, 200 cc.; 11.30, 300 cc.; 1 p.m., 200 cc. ... | 700 | 70 |
| G. | 6.30 a.m., 200 cc.; 11.30, 300 cc.; 1 p.m., 400 cc. ... ... ... | 900 | 90 |
| N. | 6.30 a.m., 200 cc.; 8.45, 400 cc.; 12 p.m., 300 cc. ... ... | 900 | 90 |
| P. | 6.30 a.m., 400 cc.; 11.30, 500 cc. ... ... ... ... ... | 900 | 90 |

Chart I. *European subject (mixed diet, total nitrogen 10 g.).*



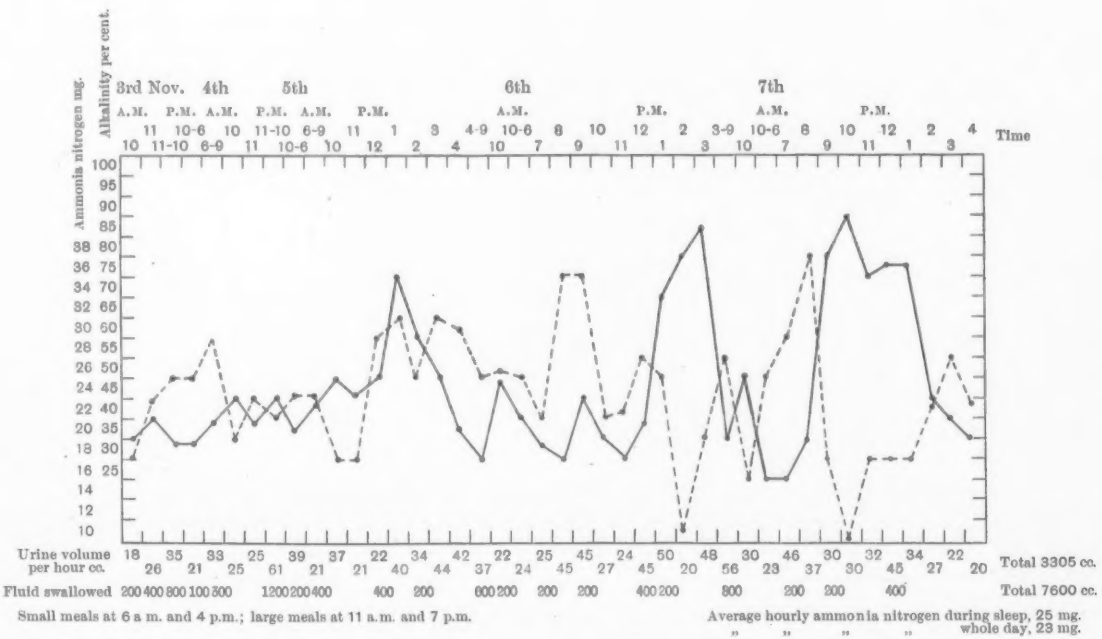

Curves for alkalinity per cent. (continuous line) and for ammonia nitrogen (broken line) of the urine.

Chart II. *Hindoo subject (vegetarian, total nitrogen 5 g.)*




Curves for alkalinity per cent. (continuous line) and for ammonia nitrogen (broken line) of the urine.

Chart III. *Tamil subject (mixed diet, total nitrogen 8 g)*


Ammonia nitrogen mg.
Alkalinity per cent.
3rd Nov. 4th 5th 6th 7th
A.M. P.M. A.M. P.M. A.M. P.M. A.M. P.M. A.M. P.M.
Time
Urine volume per hour cc.
Fluid swallowed
Total 3305 cc.
Total 7600 cc.
Small meals at 6 a.m. and 4 p.m.; large meals at 11 a.m. and 7 p.m.
Average hourly ammonia nitrogen during sleep, 25 mg.
" " " " whole day, 23 mg.

Curves for alkalinity per cent. (continuous line) and for ammonia nitrogen (broken line) of the urine.

and with a total nitrogen excretion of 8 g. was from 23 mg. ammonia nitrogen per hour to 33 mg. per hour. These variations may be normal, but I do not think that such marked variations in the daily ammonia have been noted before in apparently normal individuals, on a constant and customary diet, mainly vegetarian in nature, as taken by most of my subjects. An increase was observed just as frequently in the strict vegetarians as in those using a mixed diet, the absolute quantities of ammonia being of course lower for the vegetarians. It is noteworthy that my subjects appeared to avoid an acid-producing diet.

It was considered that some tropical disease might be responsible, since the high figures resembled those for ammonia excretion in febrile diseases, as described in text books of clinical medicine. Malaria was the only disease of which a history was obtained in a fair number of my subjects; and these did not excrete large amounts more frequently than those without a history of malaria.

In Table I details of the total acidity as arranged by Palmer and Henderson [quoted by Cole, 1919] are given; the figures show that the total acidity was distinctly high in some cases, indicating an acidosis. As in Europe the acidity, generally speaking, varied directly with the total nitrogen. Also the urine was acid on a mixed diet and alkaline on a vegetable diet (see Table I, acidity, Leathes' method). In Europe the volume of the urine is stated to vary inversely as the acidity; but in Singapore the volume more often varied directly as the acidity (see Tables II, III, IV, and Charts I, II and III). Many of the subjects showed that large quantities of water were lost by perspiration, the amount of urine voided being much less than the amount of fluid swallowed. It is evident that a good deal of acid must have been removed by sweat, since perspiration is known to lower the acidity of the urine. Diminution of sweat in a climate like that of Singapore would evidently cause an acidosis.

### The Alkaline Tide.

Recently Leathes [1919] stated "...the traditional ascription of the alkaline tide to the secretion of hydrochloric acid in the stomach appears to be erroneous. An intense alkaline tide is normal in the morning when no food is taken; alkaline tides after other meals than the first in the day are certainly not the rule, and in the experience of the writer do not occur. It is not even *a priori* probable that they should, since the passage of acid from the stomach is the signal for the secretion of alkaline digestive juices into the small intestine and for absorption into the blood from the intestine to begin." Leathes holds that the matutinal alkaline tide is accounted for by depression of the respiratory centre during sleep and increased activity on waking and some time later, so that in this way acid is removed by the lungs and not by the urine to the usual extent.

My results in Singapore were quite different from those of Leathes, and

certainly confirmed the traditional ascription of the alkaline tide to the secretion of hydrochloric acid. I found that an alkaline tide, although not constant, was the rule about three hours after any meal in Singapore. I employed the method for estimation of alkalinity and acidity described by Leathes in his paper, the reaction of the urine being given as so much per cent. alkali or so much per cent. acid. The subjects were selected from those employed in the first part of this research. Some of them took meals as in Europe about 8 a.m., 1 p.m., and 7.30 p.m., with light tea (tea, bread and butter) about 6.30 a.m. and 4.30 p.m.; others, as was their custom, took large meals about 11 a.m. and 7 p.m. with small meals about 6 a.m. and 4.30 p.m.

My figures for subjects under normal conditions may be summarised as follows. In 100 cases a matutinal tide was possible; in 68 cases it was present (68 %); in 32 cases it was absent. In 78 cases a tide was possible after the midday meal and evening meal; in 57 it was present (73 %); whilst in 34 out of these 57 occasions the tide was better marked than the corresponding matutinal tide. These points are illustrated in Charts I, II and III, and in Tables II, III and IV. It will be observed that the tides appeared also in those subjects who excreted large amounts of ammonia. In these Tables and Charts I have stated the quantities of fluid swallowed and the quantities of urine voided.

In another series of experiments no food was taken in the morning before 11 a.m. Although in some cases the matutinal tide still appeared, it was absent in the majority (80 %) of cases. On the other hand Leathes found that a tide is still present if no food is taken in the morning. It is well known that acid is sometimes present in the stomach when there is no food there. It is likely that acid might appear at the customary time, if food be only occasionally omitted, and thus cause an alkaline tide.

It was noted that in a European subject in Singapore the tide was often absent after lunch if work was commenced soon after the meal; but it was present if two hours' interval was taken. This was the custom for the Asiatics, who rested between 11 a.m. and 1 p.m., taking lunch between 11 a.m. and 12 noon.

I repeated Leathes' experiments with hyperpnoea and found that hyperpnoea readily made the urine alkaline as he points out; but hyperpnoea was not responsible for the tides in Singapore, some of my subjects breathing quietly and slowly all day, in fact being half asleep during the experiment.

My results resembled the conclusions of Hasselbalch whom Leathes quotes: "After each meal the urine was without exception more acid than before, but showed the lowest acidity about three hours later." It seems that the secretion of alkaline saliva renders the urine more acid just after a meal and that the secretion of hydrochloric acid renders the urine more alkaline about three hours after a meal, seeing that it is at this time the acid reaches its maximum in the stomach.

I found that, as a rule, the smallest amounts of ammonia were excreted

three hours after a meal, so that the ammonia may be required in the stomach at that time. It seems likely that this is so from the following theory recorded by Macleod [1918]. The stomach mucosa is particularly rich in ammonia which, it is supposed, carries out the hydrochloric acid into the cavity of the stomach, the ammonia passing back again to the cells of the mucosa. Therefore it seems that most ammonia would be required in the stomach three hours after a meal, when the acid secretion is at its maximum. Schittenhelm and A. Loeb and Gammeltoft [quoted by Hammarsten and Hedin, 1914, p. 767] have also observed a fall in the ammonia elimination a few hours after a meal and *vice versa*.

## Note on Amino-acid Excretion.

The amino-acids were estimated by subtracting the result obtained by Folin's method of estimating ammonia, from that obtained by Malfatti's method.

Table I shows that, with the exception of Tamil IV, the amount of amino-acid nitrogen varied directly with the total nitrogen, being about 0·12 g. with a total nitrogen of about 8 g., and about 0·07 g. with a total nitrogen of about 5 g. In Tamil IV the amino-acid nitrogen was 0·10 g., the total nitrogen being 5·02 g. His ammonia nitrogen was also relatively high. When large amounts of ammonia were excreted the amino-acids were sometimes increased; at other times they were not altered.

The average amino-acid nitrogen per cent. of total nitrogen was about 1·2 with a total nitrogen of about 8 g., and about 1·5 with a total nitrogen of about 5 g.

## Summary.

1. Further observations were made to endeavour to determine the cause of the frequent excretion of relatively large amounts of ammonia in Singapore, as estimated by Folin's and Malfatti's methods. Some results regarding amino-acid excretion and the alkaline tide were also obtained.

2. The hourly excretion of ammonia in subjects excreting a normal amount of this substance, varied, as a rule, directly as the acidity or inversely as the alkalinity. This supports the theory that urinary ammonia is used to neutralise acid.

3. The hourly variations for those excreting large amounts of ammonia resembled those for individuals excreting normal amounts. Any subject was likely to show an increase, which lasted sometimes for a day, sometimes for several days. The increased excretion was spread out over the whole day and was not confined to the hours of greatest exposure to the effects of the climate. When normal amounts were excreted a similar condition existed. Nevertheless, it is considered that the climate was responsible for the acidosis since the largest daily amounts of ammonia, noted in all subjects, were excreted by the individuals who were most exposed to its effects. It is noteworthy that my subjects appeared to avoid an acid-producing diet.

4. Well-marked alkaline tides, although not constant, were the rule three hours after any meal, being just as frequent after the midday and evening meals as after the morning meal. The traditional ascription of the alkaline tide to the secretion of hydrochloric acid in the stomach was therefore supported.

5. The ammonia excretion in the urine varied inversely as the acidity of the stomach. This supports the theory that ammonia is used to carry hydrochloric acid from the stomach mucosa to the stomach cavity.

6. In Singapore the volume of the urine varied directly as the acidity, in this respect differing from Europe. In some cases, the total acidity was distinctly high, indicating an acidosis.

7. The amino-acids in the urine varied directly as the total nitrogen.

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# LIV. CUORIN.

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(*Received July 17th, 1920.*)

The name cuorin was given by Erlandsen [1907] to a new lipin substance which he isolated from the ether extract of heart muscle. In many of its properties cuorin was very similar to kephalin, but differed from this substance in the relative amounts of nitrogen and phosphorus present in the molecule. It formed one of a constantly increasing group of alleged new lipins isolated chiefly by the German school about a decade ago. At that time it seemed as if the number of different lipins present in the tissues was very great, while their complexity of composition and the difficulties experienced in isolating them made this interesting field of biochemistry appear to be almost hopelessly difficult. Gradually, however, as the result of further research, it became obvious that many of these alleged new bodies had no claim to be regarded as unit substances and were largely mixtures or decomposition products of well-known lipins. Thus MacLean [1918] was able to show that the number of phosphatide lipins present in animal tissues could with certainty be reduced to four—lecithin, kephalin, sphingomyelin and cuorin—but that even this short list was probably too long, since the evidence for the existence of cuorin as a chemical entity was meagre in the extreme. In this connection he remarked that there was no definite proof that the substance was not a mixture, and that though undoubtedly a substance resembling Erlandsen's cuorin could be obtained from tissues, yet the conditions under which this substance was isolated did not exclude the possibility of the body being an artificial product. This opinion was recently substantiated by Levene and Komatsu [1919], who, in a very fundamental investigation into the structure of kephalin, came to the conclusion that cuorin was a mixture composed chiefly of kephalin with certain degradation products.

A review of the literature disclosed the fact that many of the cuorin-like substances isolated differed materially in their nitrogen to phosphorus ratio, and that while some of them had a N : P ratio of 1 : 2, in others this ratio was as high as 1 : 1·5 or even more. All this lent force to the view that cuorin was probably a substance which did not exist in fresh tissue as such, but was formed as the result of the various manipulations utilised in the extraction of the lipins. On this hypothesis an attempt was made to isolate the lipins *from*

*very fresh tissues as rapidly as possible*, using the purest of solvents, in the hope that decomposition would be largely prevented. If cuorin were a decomposition product it should be possible to prevent its formation, and no substance of this nature should then be found in the tissue. By very careful and rapid manipulation of heart muscle, we found that the substance which corresponded to Erlandsen's cuorin had a N : P ratio of nearly 1 : 1 instead of 1 : 2 and was really slightly impure kephalin. It is, therefore, certain that cuorin is an artificial product and has no further claim to be regarded as a definite lipin. This observation simplifies to a considerable extent the classification of the lipins, for it is now reasonably certain that the tissues contain only three phosphatides—lecithin, kephalin and sphingomyelin—and that the addition to these phosphatides of the two cerebrosides—phrenosin and kerasin—completes the list of lipin substances as we know them to-day. Although the constitution of all these bodies has not yet been definitely ascertained, much light has recently been thrown on the question, and soon we may hope to know as much of the structure of the lipins as we do of such a well-defined group as the carbohydrates.

## Experimental.

Ox hearts, obtained immediately after death, were freed from macroscopic fat and passed through a mincing machine. The resulting material was dried by stirring it up for a short time in a fairly large volume of pure acetone; the mixture was then strained through a piece of muslin, bound up in sailcloth, and freed as much as possible from acetone by the use of a powerful press. The tissue, which was now almost dry, was broken up and spread on a glass plate under a fan for about an hour during which it was constantly broken up with a spatula. It was then passed through a coffee mill and a fine powder obtained. The whole process was carried through as expeditiously as possible, so that the tissue was obtained in the form of a fine dry powder ready for extraction, a few hours after the death of the animals. In our opinion, this is most important, as there is little doubt that decomposition often takes place while the tissue is being dried, especially when this is done more or less slowly at a moderately high temperature, as is often the case.

The next step was to extract the powder with absolute alcohol in the usual way until the extract contained little or no residue on evaporation of the alcohol. After thorough treatment with alcohol the tissue was finally extracted with ether.

The alcohol extracts were mixed, the alcohol removed at ordinary temperature by means of a vacuum pump, and the thick syrupy mass remaining taken up with pure ether. Insoluble substances were removed by the centrifuge and the ether extract treated with excess of acetone. This process was repeated several times. The final precipitate was purified by water and acetone [MacLean, 1912], and the waxy substance obtained dried *in vacuo*. By further treatment with ether followed by centrifuging, some remaining

sphingomyelin was separated, and ultimately a substance was obtained which gave a clear solution in ether. This ethereal solution was again treated with acetone and the precipitate dried *in vacuo* over sulphuric acid. The dry material was then thoroughly shaken up with excess of alcohol, when part of it remained insoluble. The alcohol-soluble fraction consisted of more or less pure "lecithin," and on analysis gave N 1·97 % and P 4·05 %; N : P = 1 : 1·07.

The alcohol-insoluble portion should of course contain kephalin and Erlandsen's cuorin. After dissolving in ether and reprecipitating with acetone it gave on analysis 1·6 % of N and 4·1 % of P. This substance was then treated exactly according to Erlandsen's directions for separating cuorin from kephalin, when a small amount of a body corresponding in solubility and physical properties to Erlandsen's cuorin was obtained. This product which, if it were cuorin, should have a N : P ratio of 1 : 2 gave on analysis 4·06 % P and 1·66 % N, *i.e.* a N : P ratio of 1 : 1·1. The substance was therefore more or less impure kephalin, and no evidence of the presence of a substance of the composition of cuorin could be obtained. It would thus seem that cuorin is only found under conditions where decomposition is liable to take place and is really a mixture containing some decomposition products.

Subsequent treatment of the alcohol-extracted tissue with ether showed that practically all the lipin material present was removed by the alcohol. The ether extract contained only mere traces of phosphatides, and no body of the nature of cuorin was present.

## Conclusions.

The cuorin described by Erlandsen is not a chemical unit, but results from decomposition taking place in the tissues during the processes utilised for the extraction of the lipins. This decomposition is probably most active during the drying of the tissue. When precautions are taken to dry the perfectly fresh tissue very quickly by means of acetone, no cuorin is obtained.

The only phosphatides present in animal tissues are lecithin, kephalin and sphingomyelin.

The expenses involved in this work were defrayed from a grant for which we are indebted to the Royal Society.

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# LV. FACTORS INFLUENCING ALKALOIDAL CONTENT AND YIELD OF LATEX IN THE OPIUM POPPY (*PAPAVER SOMNIFERUM*).

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(*Received July 21st, 1920.*)

## I. Introduction.

The present paper is a summary of a full account of the work of the writer and others which is being published elsewhere[1]. A very large amount of data has been accumulated which it is hoped may be of interest to physiologists but under present conditions it is impossible to reproduce all the figures here. Typical figures only will therefore be given in the present paper.

The work represents an investigation arising out of war conditions. In pre-war days the bulk of the opium required for alkaloidal manufacture and for medicinal purposes was provided by Asia Minor, the Balkans and Persia. The morphine content of Indian opium was too low to enable it to compete in that market. With the entry of Turkey into the war supplies of medical opium were threatened and it became imperative to make use of Indian opium. The writer was placed on special duty in October 1916 to investigate the reason for the low morphine content of Indian opium. The work met with success and there is no reason why India should not put opium on to the market of at least as high morphine content as the product of Asia Minor and the Balkans.

Fortunately for the work the writer has had the advantage of collaboration with Mr H. M. Leake, M.A., Economic Botanist to the Government of the United Provinces. Mr Leake had for several years previously been endeavouring to isolate races of poppy which would be resistant to the Blight disease (*Peronospora arborescens*) which causes very serious havoc annually in India. He was therefore able to supply seed of pure races of poppy for experimental work at once. Much of the work which has been carried out on drug plants appears to suffer from the fact that pure races of plants were not used. In addition to the use of pure races the work has been carried out on a large scale and at several centres, *e.g.* in the plains and in the Himalayas. Some five or six acres of the same pure race have been under experiment annually.

[1] *Memoirs of the Dept. of Agric. in India.*

## II. Description of the Method of Extraction of Latex.

It will make the description of the work easier if the method of extraction of opium as practised by the Indian cultivators is first described. The operation is commenced some 10 to 20 days after the fall of the flower petals. The capsules are considered to be ready for lancing as soon as they feel firm to the touch and are yet green. The instruments used are:

(1) A knife which consists of two to four parallel sharp pointed blades bound together with cotton, the binding being so done that the points of each blade are about one-twentieth of an inch apart and all in the same plane.

(2) A small iron scoop.

(3) Unglazed earthen pots in which to store the opium.

The cultivator then usually divides his fields into three portions *A*, *B*, *C*. The lancing is invariably commenced just after midday and is carried out by men, women and children. They begin at the edge of the field and work backwards as otherwise the exuding latex would be brushed off on their clothes. The hand is quickly passed over the capsules. If a capsule is considered to be ready it is grasped in one hand and the knife is drawn vertically upwards over its surface from just above the stalk to just below the stigmatic rays at the top of the capsule. Sometimes the cut is made in the reverse direction, *i.e.* downwards. The second finger of the hand holding the knife is placed near the points of the knife on the surface of the capsules to steady the motion of the knife. Great care is taken not to cut too deeply into the capsule. If the cut be made too deep, then the wall of the capsule may be cut completely through with the consequent secretion of the latex on the inner surface. Good operators can incise 150 to 200 capsules in an hour.

Immediately on incision the latex commences to flow. It varies in colour from milk white and smoky white through pale pink to a very bright pink. It rapidly begins to darken however. Next morning at 6 a.m. or later if there is a heavy dew, the opium is collected. It is scraped off the cut surface with the blunt edged iron scoop. From the scoop it is transferred to the earthen pots. Usually the cultivator lances only one-third of his field (*e.g.* portion *A*) on the first day. After collecting the opium next morning from that portion he then lances portion *B*, that is on the second day. On the third day portion *C* is lanced. On the fourth day he again returns to lance portion *A*. On this occasion many more heads are now ready for a first lancing. In addition a second lancing is given to those capsules which were lanced on the first day. On the fifth day portion *B* receives its second lancing and on the sixth day *C* receives its second lancing. On the seventh day *A* is lanced for the third time and on this occasion more heads are usually ready for a first lancing. Those already lanced once now receive a second lancing and those already lanced twice receive a third lancing. Portions *B* and *C* come in for similar treatment in regular rotation. The lancings are continued as long as the capsules continue to give appreciable yield of latex. Usually each field is lanced

four or five times and occasionally as many as eight times so that some heads would then have received eight lancings. In some poor seasons and on some poor crops the heads cease to yield opium after the second lancing. The whole opium produce of each field is then mixed together and, after three to six weeks' storage, is brought to an official of the Government Opium Department by whom each cultivator's opium is weighed and examined for consistency or dry matter by touch and for certain adulterations (*e.g.* starch by the iodine test). The opium officers reach such a high degree of skill that they can usually tell the moisture content of the opium by touch to within 2 or 3 %. The cultivator at once receives the value of his opium except for a small balance which is paid some two months later when the district officer's examination has been checked by analysis at the Government Opium Factory, to which place all the opium is sent.

### III. The Alkaloidal Content and Yield of Latex from each of a series of Successive Lancings of the same Capsules.

We have established the fact that on the first occasion on which a capsule is incised by the cultivator the opium is far richer in morphine than is the produce of the second lancing and this again than that of the third lancing and so on. Cases have been met with in which the opium obtained from the fifth lancing gave no morphine at all by the method of estimation laid down in the *British Pharmacopoeia*. The following analysis is typical of many hundreds of estimations we have carried out of the morphine content of the opium from a series of successive lancings of the same capsules.

| | No. of lancings | | | | | |
|---|---|---|---|---|---|---|
| | first | second | third | fourth | fifth | sixth |
| Morphine percentage on dry matter of opium | 13·9 | 9·6 | 5·8 | 3·6 | 2·2 | 1·6 |

We have met with no case in which the opium of a particular lancing was of higher morphine content than the opium of a previous lancing of the same capsules.

As regards the yield of latex at each successive lancing it is difficult to state typical figures. Thus in the hills one usually finds that the capsules will bear a larger number of successive lancings than they will in the plains so that as a rule in the hills the first three or four lancings may each produce about the same amount of opium. In the plains, however, one usually finds the first lancing of a particular capsule produces the highest yield of latex and the yield falls off with each subsequent lancing, provided there are no sudden changes in the weather conditions. The following figures are perhaps typical.

*Table showing yield of latex (dried at* 100° C.) *at each successive lancing.*

| | Yield of latex in g. dry matter per 1000 capsules No. of lancings | | | | | |
|---|---|---|---|---|---|---|
| Locality | first | second | third | fourth | fifth | sixth |
| Elevation 3500 feet (Himalayas) ... | 20·6 | 33·8 | 29·5 | 18·2 | 5·6 | 0·5 |
| Cawnpore ... ... | 35·5 | 12·4 | 5·4 | 2·1 | 0·4 | 0·1 |

Varying weather conditions, as will be shown later, exert very great influence on the yield on any particular day. Thus cloudy weather or East winds usually considerably reduce the yield. The author has found similar phenomena in his work on sugar yielding palms [1913].

The rapid fall in morphine content of the latex from each successive lancing, raises the question as to whether any substance is formed in place of the morphine in the later lancings. Indian opiums are particularly rich in codeine and it seemed probable as this alkaloid is so closely related to morphine in its constitution, that one would find an increase in the codeine content of each successive lancing. This however is not the case. Narcotine is the only other alkaloid present in sufficient quantity to be of interest in this connection but here again there is, as a rule, no rise in narcotine content in the later lancings. The following figures show the percentages of morphine, narcotine and codeine in the dried latex of the first four successive lancings of the same capsules.

| Number of lancing | Percentage of alkaloids in dry matter of latex | | | |
|---|---|---|---|---|
| | Morphine | Narcotine | Codeine | Total of morphine, narcotine and codeine |
| First ... | 11·18 | 6·85 | 2·25 | 20·28 |
| Second ... | 9·28 | 6·34 | 2·71 | 18·33 |
| Third ... | 6·38 | 4·18 | 2·94 | 13·50 |
| Fourth ... | 3·41 | 3·36 | 3·64 | 10·41 |

As previously stated one always finds the same rapid progressive decrease in morphine content in each successive lancing. As regards narcotine and codeine one does not observe the same regularity in behaviour. Codeine certainly usually shows a slight increase just as in the table. On the other hand narcotine sometimes increases in the later lancings. We are investigating this matter in greater detail. It may be stated here however that the total nitrogen content of the dry matter of the latex from each successive lancing remains practically constant, so that some nitrogenous substance takes the place of morphine in the subsequent lancings. At present our work indicates that the substance is not an alkaloid.

It seemed of great interest to discover if decreasing or increasing the interval of time between the successive lancings would affect the rate at which the morphine content of the later lancings falls off. The following intervals of time between the successive lancings were experimented with:

(1) 4 hours, (2) 1 day, (3) 2 days,
(4) 3 days, (5) 4 days, (6) 5 days.

Our results showed that when the interval between successive lancings was one day or more the morphine content of the opium of each successive lancing fell away in exactly the same manner whether that interval was one, two, three, four or five days. With an interval of only four hours between the successive lancings indications were obtained that the falling off in morphine

content of each successive lancing was not so marked. The bearing of this phenomenon will be discussed in the last section of this paper. Strong indications were obtained that after receiving the first lancing, the capsules require a certain minimum interval of time before the second lancing in order that they may give a free yield of latex at the second lancing.

## IV. The Variation in Rate of Flow and Morphine Content of the Latex at Different Periods of Time after Incision.

Numerous experiments were performed in which latex was collected in fractions at various intervals of time after incision of the capsules. Thus immediately on making the incision latex flows out. This was collected within one minute of making the incision. From the same incised surface latex continues to flow and collections were made at certain set times during the ensuing 16 hours after which time we have only on rare occasions observed any flow of latex from an incised surface. In the latex of each successive fraction there is again a steady fall in the morphine content. Moreover when some two days later a second incision is made on the capsule and the latex again collected in fractions the fall in morphine content again takes place in each successive fraction, the first fraction usually showing about the same morphine content as the final fraction of the previous lancing. Latex collected in fractions from a third successive incision behaves in an exactly similar manner. The table gives a typical result.

| | | Percentage of morphine in latex dried at 100° C. Time of collection | | | | |
|---|---|---|---|---|---|---|
| No. of lancing | Date of lancing | 9 a.m. (immediately on incision) | 12 a.m. | 3 p.m. | 6 p.m. | 6 a.m. (next morning) |
| 1 | 13–4–19 | 12·3 | 11·0 | 10·9 | 10·1 | — |
| 2 | 16–4–19 | 9·7 | 7·8 | 7·7 | 5·9 | — |
| 3 | 19–4–19 | 7·3 | 5·7 | — | — | 5·5 |

The writer considers that these figures indicate that morphine is stored principally in the capsule. When the capsule is lanced the latex first flowing out would therefore be of maximum morphine concentration. As the flow continues the latex would flow in from parts of the plant below the capsule, where the morphine is presumably present in lower concentration. The results also tend to disprove the theory that morphine does not occur as such in the plant [Winterstein and Trier, 1910, True and Stockberger, 1916]. Figures showing the rate of flow of latex at various intervals of time after incision are given in the main publication. It is sufficient to state here that 22·6 to 60·6 of the total yield of latex may be exuded within a minute after incision.

## V. The Effect of Different Systems of Lancing on the Yield and Composition of the Latex.

The methods of lancing in use in the various opium producing centres differ greatly. In Asia Minor and the Balkans one spiral cut is made round the capsule and no subsequent lancing is carried out on the same capsule. In Egypt transverse cuts are made. We have already stated that in India one vertical cut with a multiple bladed knife is made at each lancing. It seemed desirable in view of the high morphine content of the latex yielded at the first lancing, to obtain as high a yield as possible at the first lancing. With this end in view experiments were carried out in which two, three, four and five and even six vertical cuts with a four bladed knife were made at the first lancing. A very definite result was obtained from these experiments in that if one obtains a large yield at a first lancing by increasing the number of incisions, then the percentage of morphine in the latex diminishes. Following up this matter, further experiments were made in which only one incision, a very tiny one was made into the capsule so that only a small yield of opium was obtained. The opium obtained in this manner was of distinctly higher morphine content than that obtained by making incisions of the usual length.

Further when only a tiny incision is made at each lancing then the rate of fall in morphine content of the opium of each successive lancing is much less rapid.

Some interesting data have been accumulated both in field and pot experiments on the connection between the yield of latex and the number of incisions made in the capsule. The following table gives the result of a field experiment in which some 5000 capsules were taken for experiment. Some of them were lanced with a single vertical incision, some with three incisions and the remainder received a minimum of five incisions per capsule. In each case a four bladed knife was used. The first two groups received subsequent lancings also but only the results of the first lancing are given in the table.

*Effect of increasing the number of incisions at each lancing on the yield of latex per capsule.*

| | *A* 1 vertical incision | *B* 3 vertical incisions | *C* 5 or more vertical incisions |
|---|---|---|---|
| Gms. latex (dried at 100° C.) per 1000 capsules | 30·3 | 40·9 | 20·9 |

Thus by increasing the number of incisions per lancing from one to three a slight increase in yield of latex is obtained. When however the number of incisions per capsule is increased to five or more then the yield of latex per capsule is considerably diminished. These results were fully borne out in an experiment on poppy plants grown in pots. In this case experiments were made on capsules receiving one, two, three and six incisions at each lancing. The table shows the yield of latex dried at 100° C., per capsule for the first lancing only.

*Yield of opium per capsule as affected by using one, two, three and six incisions at each lancing. (Pot experiment.)*

| | Number of incisions at each lancing | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 6 |
| Gms. dry opium per capsule, 1st lancing only | 0·0294 | 0·0352 | 0·0374 | 0·0277 |

Here again it is seen that increasing the number of incisions does not result in a corresponding increase in the yield of latex, and beyond a certain point may cause a decrease.

The ordinary lancing knife as used in India contains four blades the points of which are set about $\frac{1}{20}$th inch apart. We have carried out experiments to determine the comparative effect of using knives with two, four and six blades. Increasing the number of blades does not increase proportionally the yield of latex. The six bladed knife in our experiments gave about a 20 % increase in yield of latex over the yield obtained with the two and four bladed knives which gave practically identical yields.

## VI. The Yield and Alkaloidal Content of the Latex from Different Capsules of the same Plant.

One may find as many as 40 capsules in various stages of development on a plant of the opium poppy. Usually, however, a plant has one or two or perhaps three capsules. The oldest is at the end of the main stem and will be referred to as the terminal capsule. The subsequent capsules are borne each at the end of a branch arising from an axil of a leaf on the main stem and the younger the capsule the farther down the main stem does its branch originate. These will be referred to as lateral capsules. We have found as a result of a large number of experiments carried out on 50 pure races of poppy plants that the oldest or terminal capsule produces opium of much higher morphine content than do the younger capsules and it appears that the morphine content of the latex diminishes progressively the later the order of origin of the capsule. It must be clearly understood that this phenomenon has no connection with the unripeness of the later capsules. Our lancings were not carried out until the capsules were at the correct latex yielding stage. Usually also, though not invariably, the terminal capsules yield more latex than the lateral capsules.

The table gives results typical of our experiments. In the experiment to which this table refers 1620 plants each with several capsules in process of development had their terminal capsule lanced. The second, third and fourth capsule on each plant was lanced when ready. The opium obtained from the terminal and from first, second and third lateral capsules was examined separately.

| Description of capsule | No. of lancing | Date of lancing | Yield of dried opium per 1000 capsules, g. | Percentage of morphine on dried opium |
|---|---|---|---|---|
| Terminal ... | 1 | 2–3–18 | 79·7 | 14·0 |
| | 2 | 5–3–18 | 27·0 | 8·4 |
| | 3 | 9–3–18 | 8·2 | 5·3 |
| | 4 | 12–3–18 | 3·5 | 3·3 |
| 1st lateral .... | 1 | 5–3–18 | 34·6 | 12·2 |
| | 2 | 9–3–18 | 8·0 | 7·1 |
| | 3 | 12–3–18 | 2·2 | 4·9 |
| | 4 | 15–3–18 | 0·3 | — |
| 2nd lateral ... | 1 | 9–3–18 | 13·7 | 10·1 |
| | 2 | 12–3–18 | 6·5 | 6·1 |
| | 3 | 15–3–18 | 0·7 | — |
| 3rd lateral ... | 1 | 12–3–18 | 14·3 | 8·0 |

In an examination of the latex from the terminal and lateral capsules of 50 pure races of poppy not a single case was met with in which the morphine content of the latex from the lateral capsules was higher than that of the latex of the terminal capsules.

Experiments were carried out to discover if by leaving the terminal capsules unlanced one would obtain latex of higher morphine content from the lateral capsules. It was found that the morphine content of the latex of the lateral capsules was not increased by leaving the terminal capsules unlanced.

Indications were however obtained which showed that by removing all lateral buds a large yield of latex could be obtained from the terminal capsules.

## VII. The Relation between the Stage of Development of the Capsule and the Yield and Alkaloidal Content of its Latex.

It has already been shown in Section III that the morphine content diminishes progressively in the latex from each of a series of successive lancings from the same capsules. As will be shown here this phenomenon has nothing whatever to do with alterations in morphine content of the latex with increasing age of the capsule as at first sight might appear possible. We have carried out experiments on this point during three seasons with identical results in each season. Some 20,000 plants have been included in each year's experiments.

Capsules have been lanced at the following intervals of time after flowering: 5, 9, 13, 17, 21, 25 and 29 days, *i.e.* at all stages from soft green to the ripe stage. Controls have been carried out by lancing side by side with the experimental capsules on each day a number of capsules in the same field which were considered by the cultivators to be ready for lancing. The results show that if the capsules are lanced only four or five days after flowering then the morphine concentration of the latex is distinctly low. About nine days from flowering the latex reaches its maximum morphine content and this maximum is maintained at whatever age beyond nine days the capsules are first lanced.

The yield of latex is low when the capsule is very immature but rises to a maximum when the capsules are first lanced at 15 to 17 days from flowering. When the first lancing is carried out later than this the yield of latex again diminishes. The table summarises the results for 1918–19 which are typical of those of the other seasons.

*Table showing connection between stage of development of capsule and the yield and morphine content of the latex.*

| No. of days between flowering and first lancing | First lancing: Latex dried at 100° C. | | Second lancing (2 days later): Latex dried at 100° C. | |
|---|---|---|---|---|
| | Percentage of morphine | G. per 1000 capsules | Percentage of morphine | G. per 1000 capsules |
| 5 | 10·2 | 27·7 | 6·5 | 23·0 |
| 9 | 11·7 | 28·2 | 8·4 | 19·7 |
| 13 | 12·1 | 30·4 | 8·3 | 26·1 |
| 17 | 11·4 | 36·9 | 8·7 | 21·1 |
| 21 | 12·5 | 26·3 | 10·7 | 22·1 |
| 25 | 12·5 | 22·1 | 9·1 | 16·9 |
| 29 | 12·2 | 25·3 | 10·4 | 8·4 |

## VIII. The Effect of Climate and Weather Conditions.

The literature records the results of numerous experiments carried out with the idea of tracing the influence of climate and weather conditions on drug production in various medicinal plants. This work is summarised in our main publication. As regards our own particular problem, the discovery of the reason for the low morphine content of Indian opium, it may be stated at once that climate as such does not control to any important extent the power of the poppy plant to produce morphine. Opium of high morphine content has been produced in every continent, in climates as different as those of the tropics and Sweden.

Our work in this connection has been carried out during four seasons, on a very large scale. We have been fortunate in being able to carry out trials with the same pure race in various places in the plains of northern India and in the same season at altitudes of 4000, 5000 and 6500 feet in the Himalayas. At the latter altitude our poppy was at times under snow. Our remarks apply especially to morphine. As regards other alkaloids our investigations are not so far advanced. Codeine for instance is always high in Indian opium. We have examined the produce of numerous pure races of Indian opium selected by Mr Leake and we have never found less than about 1·8 % codeine. Some samples yielded over 4½ % and the average for Indian opium we would put at about 3 %. Turkish opium only contains about 0·25 %.

Narcotine is usually present in opium in quantities amounting to 5 to 8 % but French opium has at times been reported to contain none of this alkaloid [van Ittalie and Kerbosch, 1911]. The matter is referred to at a greater length in the complete account of our work.

*Effect of Climate.*

Before dealing with our experimental results it will be advisable to point out that previous investigators on opium have not recognised the large difference which exists in the morphine content of the opium of each successive lancing, nor have they realised the variation in morphine content of the opium produced by the various capsules of the same plant. In our work on climatic conditions we have only used the terminal capsules of each plant as a source of opium and we have kept separate the product of each successive lancing. At each centre we have usually had ½ an acre of poppy at least, while at our main station at Cawnpore five to six acres of the same pure race have been under experiment. On each area very large numbers of samples were collected amounting to some hundreds at Cawnpore. Much attention has been paid in our work to the determination of the range of variation which occurs over a field which is all under similar treatment. Over a given field provided only terminal capsules are used the various samples of opium of the first lancings usually show quite a small range of variation say from 11 to 13 % morphine for a race averaging 12 % of morphine.

The following table summarises the figures obtained at the various stations in 1916–17, 1917–18, 1918–19 and 1919–20.

*Table showing effect of climate or locality on the morphine content of the opium. Produce of first incision of terminal capsules only.*

| No. of samples examined | Locality | Percentage of morphine calculated on opium dried at 100° C. | |
|---|---|---|---|
| | | Range of variation | Average |
| | 1916–17 | | |
| 10 | Sitoli (Alt. 5000 feet) ... ... | 10·9–13·9 | 12·7 |
| 1 | Douglas Dale (Alt. 4000 feet) ... | — | 10·6 |
| 1 | Chaubattia (Alt. 6500 feet) ... | — | 10·3 |
| 1 | Cawnpore ... ... ... ... | — | 11·1 |
| | 1917–18 | | |
| 91 | Cawnpore ... ... ... ... | 11·8–15·8 | 14·0 |
| 1 | Fatehgarh (Ankin) ... ... | — | 15·1 |
| 2 | „ (Mianganj) ... ... | 12·6–13·1 | 12·8* |
| 5 | Rae Bareli ... ... ... ... | 11·2–13·4 | 12·2* |
| 11 | Douglas Dale (Alt. 4000 feet) ... | 11·3–16·7 | 13·3 |
| 1 | Sitoli (Alt. 5000 feet) ... ... | — | 10·0 |
| 2 | Chaubattia (Alt. 6500 feet) ... | 16·1–16·3 | 16·2 |
| | 1918–19 | | |
| 119 | Cawnpore ... ... ... ... | 10·5–15·8 | 12·6 |
| 12 | Rae Bareli ... ... ... ... | 9·2–12·7 | 11·1 |
| 8 | Douglas Dale (Alt. 4000 feet) ... | 9·3–12·4 | 11·1 |
| | 1919–20 | | |
| 71 | Cawnpore ... ... ... ... | 14·0–17·7 | 15·4 |
| 8 | Rae Bareli ... ... ... ... | 11·5–15·0 | 13·1 |
| 1 | Fatehgarh ... ... ... ... | — | 16·6 |
| 2 | Etawah ... ... ... ... | 16·1–17·6 | 16·8 |
| 3 | Douglas Dale (Alt. 4000 feet) ... | 13·2–15·3 | 14·0 |

* These samples were mixtures of first and second lancings and would therefore be lower in morphine content than first lancings only.

It should be stated that the pure race grown in 1916–17 was a different one from that used in the subsequent three seasons. In 1916–17 the poppy producing the one sample at 6500 feet altitude was at one stage under snow.

It would not appear that climate is an all important factor in determining the power of the plant to produce morphine. At the same time during the past three seasons in which we have been very carefully studying the behaviour of a pure race of poppy grown at various stations in the plains, indications have been obtained that locality does exert a certain amount of influence on the morphine producing power of a plant. Thus at Cawnpore during the season 1919–20 our main pure race gave an average of 15·4 % of morphine in the opium of the first lancing. At Etawah and Fatehgarh it has given perhaps a slightly better result, whereas in Rae Bareli it has only produced about 13 % of morphine. In the previous season we noticed that the Rae Bareli crop behaved in a similar manner. The climate of all these places shows no marked difference and they are all within 100 miles of Cawnpore. It is hoped to follow up this matter further.

### *The Influence of Season.*

There is evidence that the morphine content of the opium produced by the same pure race may vary from season to season. We have grown a collection of pure races for three successive seasons and have found a distinct variation in the morphine content in successive seasons. Since only one analysis was carried out in each of these cases in each season it is hardly fair to generalise from these results. We have, however, grown a large area of one pure race at Cawnpore on the same farm during four successive seasons, and numerous analyses were made in each season. The table summarises the results of the four years' experiments.

*The seasonal variation in morphine content of opium produced by the same pure race of poppy. Produce of first lancings only.*

| Season | Area under experiment acres | No. of samples analysed | Percentage of morphine in dry matter of opium | |
|---|---|---|---|---|
| | | | Range of variation | Average |
| 1916–17 | 0·5 | 16 | 11·7–14·7 | 12·9 |
| 1917–18 | 5·5 | 91 | 11·8–15·8 | 14·0 |
| 1918–19 | 6·0 | 119 | 10·5–15·8 | 12·6 |
| 1919–20 | 1·5 | 71 | 14·0–17·7 | 15·4 |

We have accumulated similar data for the same pure race grown during three seasons at Rae Bareli in the plains and at Douglas Dale in the Himalayas.

### *Weather Conditions.*

Experiments were carried out in 1917–18 and in 1918–19 to trace any connection there might be between temperature and other weather conditions and yield or morphine content of the opium produced. A continuous record

of the temperature was kept by means of a thermograph. Observations were also made of barometric and humidity conditions but produced no results worthy of consideration here. Rainfall, direction of winds and amount of cloud were also recorded. A field of 0·5 acre in extent sown with seed of a pure race was used. The plan of experiment was to select 1000 capsules daily, which were evenly distributed throughout the field, and which the cultivators considered were ready for lancing, and to lance them. In the 1917–18 experiment 1000 capsules were so selected daily throughout the period 28th February to 26th March. The yield of opium and its morphine content were determined in each day's produce. At intervals of three days the capsules received a second and third lancing; after which the plants bearing them were pulled up to prevent confusion. The plants selected each day were labelled with cloth labels of a distinctive colour in order to simplify the finding of them on the field. Only terminal capsules were included in the experiment. The results for the first lancings only of the 1917–18 experiments are set out in the table.

*Table showing yield of opium per* 1000 *capsules and its morphine content recorded daily throughout duration of opium harvest. First lancings only.*

| Date of lancing | Gms. dry opium per 1000 capsules | Percentage of morphine on dry matter of latex |
|---|---|---|
| 28–2–18 | 52·1 | 14·9 |
| 1–3–18 | 62·1 | 14·5 |
| 2–3–18 | 60·3 | 14·9 |
| 3–3–18 | 53·1 | 14·9 |
| 4–3–18 | 60·1 | 14·6 |
| 5–3–18 | 61·1 | 14·5 |
| 6–3–18 | 64·5 | 13·9 |
| 7–3–18 | 53·7 | 14·2 |
| 8 3–18 | 51·3 | 13·6 |
| 9–3–18 | 50·4 | 14·0 |
| 10–3–18 | 43·2 | 14·2 |
| 11–3–18 | 45·4 | 14·1 |
| 12–3–18 | 41·5 | 14·8 |
| 13–3–18 | 33·0 | 15·3 |
| 14–3–18 | 33·0 | 15·1 |
| 15–3–18 | 27·5 | 14·8 |
| 16–3–18 | 24·4 | 14·6 |
| 17–3–18 | 19·4 | 15·0 |
| 18–3–18 | 18·1 | 15·0 |
| 19–3–18 | 12·3 | 15·7 |
| 20–3–18 | 5·4 | 13·8 |
| 21–3–18 | 7·7 | — |

As regards morphine content it will thus be seen that this has kept remarkably constant throughout the period of opium harvest. On comparing the daily yield of latex with the temperature records it appeared that the yield of latex diminished with a rise in temperature and increased with a fall in temperature. After the 9th February, however, the latex yield fell away rapidly probably owing to the drying up of the field and the drying of the capsules and this rapid and continuous fall masked any temperature effect.

It is very interesting to note that in spite of a very rapid fall in the latex yield with advance in season, yet the morphine content of the dried latex is not modified to a detectable extent.

The 1918–19 experiments completely bore out those of 1917–18.

## IX. The Influence of Manures.

The literature provided numerous references to the effect of manuring on drug production in plants but in the writer's opinion most of the work is very unsatisfactory. Pure cultures of plants appear to have been rarely, if ever, used and it would seem that lack of experience in conducting field experiments has handicapped certain of the investigators. These matters are referred to in our main publication. We have experimented on the same pure race in the seasons 1916–17, 1917–18, 1918–19 and 1919–20.

In 1916–17, on a field of one acre, we tested the effect of $K_2SO_4$, superphosphate and nitrate of soda, alone and in all possible combinations. Each manure was applied at the rate of 400 lbs. per acre. These large amounts were put on in order to magnify any possible manurial effect. Each plot was duplicated and the usual precautions taken in field trials were used. In 1917–18, exactly the same experiments were carried out, but castor cake and cattle dung plots were also added to the series. The first two years' experiments having indicated that nitrogen was the dominant manure for poppy, in the next two seasons the trials with superphosphate and $K_2SO_4$ were discontinued and the effect of increasing amounts of $NaNO_3$ was determined. Plots receiving castor cake and poppy seed cake were also added to the series in 1919–20. The germination in 1918–19 was so uneven that the experiment was unsatisfactory in that year.

The results obtained in the seasons 1916–17 and 1917–18 were practically identical. Superphosphate and potash alone, or in combination, had no appreciable effect on the yield of latex or on the morphine content. Nitrate of soda and castor cake, however, largely increased the yield of latex but the effect on the percentage of morphine in the dried latex was almost inappreciable.

The results for 1917–18 are summarised in the table.

*Table showing effect of manures on yield of latex and its morphine content.*

| Treatment | Percentage of morphine in dry matter of latex from first lancing | Total yield of latex from all lancings as dry matter. Unmanured plot = 100 |
|---|---|---|
| No manure ... ... ... | 14·1 | 100 |
| $K_2SO_4$ ... ... ... ... | 14·9 | 103 |
| Superphosphate ... ... | 15·8 | 120 |
| $NaNO_3$ ... ... ... | 15·0 | 188 |
| $K_2SO_4$ + Superphosphate ... | 13·8 | 99 |
| $K_2SO_4$ + $NaNO_3$ ... ... | 13·8 | 192 |
| Super. + $NaNO_3$ ... ... | 14·9 | 217 |
| Super. + $K_2SO_4$ + $NaNO_3$ ... | 14·7 | 253 |
| Cattle dung ... ... ... | 14·7 | 121 |
| Castor cake ... ... ... | 14·5 | 149 |

In considering the figures one must remember that control plots showed that these variations in morphine content on the different manurial plots are less than the variations to be expected over a field uniformly treated. At the same time the influence of nitrogenous manures on the yield of latex is undoubted.

Careful records have been kept of the weights of the plants, capsules, and of the seed out-turn on each of the plots. It has been shown that these weights are proportionate to the yield of total latex on each plot. The effect of the manure is apparently therefore to produce a bigger capsule which in virtue of its size produces a larger yield of latex. For a particular race of poppy it would seem that the morphine concentration of the latex of the capsule is practically constant.

Our 1919–20 experiments were designed to test the effect of increasing amounts of nitrate of soda on the yield and alkaloid content of the latex. Plots receiving castor cake and poppy seed cake, were also added to the series.

*Table showing effect of increasing amounts of nitrogen on yield of latex and its morphine content.*

| Treatment (per acre) | Percentage of morphine in dry matter of latex | Yield of latex at first lancing as dry matter. Unmanured plot = 100 | Yield of latex as dry matter, total of all lancings |
|---|---|---|---|
| Unmanured ... ... ... ... | 14·4 | 100 | 100 |
| $NaNO_3$ 80 lbs. ... ... ... | 15·3 | 106 | 127 |
| „ 160 lbs. ... ... ... | 15·5 | 124 | 142 |
| „ 320 lbs. ... ... ... | 15·4 | 113 | 103 |
| „ 480 lbs. ..... ... ... | 16·2 | 144 | 130 |
| „ 640 lbs. ... ... ... | 16·5 | 158 | 142 |
| Poppy cake 1600 lbs. containing N = $NaNO_3$ 530 lbs. ... ... | 16·1 | 153 | 166 |
| Castor cake 1600 lbs. containing N = $NaNO_3$ 580 lbs. ... ... | 17·0 | 146 | 193 |

The morphine content of the latex shows a small but regular increase with increasing amounts of nitrogen in the form of nitrate of soda. Castor cake produced latex of the highest morphine concentration, though it did not supply as much nitrogen as the maximum amount of $NaNO_3$ applied. There is however only a difference of 2·6 % in morphine content between the latex produced on the unmanured plot and that produced on the castor cake plot. This difference is no greater than that which it is possible to find in the opium harvested from different portions of the same field uniformly treated. We are convinced that the influence is just significant however, since there were four samples of opium collected corresponding to each treatment and the analyses of these four samples agreed closely in practically every case. The figures given in the above are the averages of these analyses.

As regards yield of latex at the first lancing there has been a uniform increase in latex yield with increasing amounts of $NaNO_3$ with the exception

of the plots receiving 320 lbs. $NaNO_3$ per acre. As regards the total out-turn of opium from all the lancings the plot receiving only 160 lbs. $NaNO_3$ per acre has given as big a yield as any of the nitrate plots. The cake plots have given the biggest total yields since they kept up the yields of latex at the subsequent lancings. The important point however is that though the yield of latex has been much increased yet there is little modification in the morphine content of the latex.

## X. The Influence of Starvation.

The plants on the unmanured plots in all our manurial experiments were certainly very poor specimens and ill nourished. It seemed of interest deliberately to produce the poorest plants possible and to examine the latex produced by them. For this purpose some hard uncultivated land at Cawnpore was ploughed up and poppy grown on it in the season 1918–19. A most miserable crop resulted and we were able to obtain complete plants only some 5 to 8″ high. A portion of this field was dressed with nitrate of soda and this certainly improved to a slight extent the plants growing there but only slightly. The capsules lanced were mostly from $\frac{1}{3}$ to $\frac{2}{3}$″ long; though they were slightly larger on the plants receiving nitrate of soda.

The opium from the first lancing only from the unmanured portion yielded 8·5 % morphine. The portion receiving nitrate of soda gave opium with 10·3 % morphine. With the same pure race of poppy grown in the same season on our experimental area the extreme range of variation in the morphine content of opium from first lancings only was from 11·1 to 15·0 %. In the season 1919–20 these results were checked at Cawnpore. Around the edges of fields, owing to the land receiving poor cultivation, there are always a certain number of stunted plants which usually only produce one capsule. The smallest of these plants were selected and their terminal capsules lanced for opium. These plants were not as small as those grown specially in the previous season.

Three separate samples of opium collected yielded 12·4, 12·7 and 10·1 % morphine reckoned in the dry opium. The normal plants in the same field yielded opium containing 15 to 16 % of morphine. Similar results were obtained in experiments in the Himalayas in 1919–20. The stunted plants from the edges of the fields produced opium with 10·3 % and 7·3 % morphine respectively in the two samples examined. The normal plants in the same field produced opium with 13·2–15·3 % morphine.

Undoubtedly therefore stunted plants yield opium very low in morphine content but the stunted plants examined by us were quite abnormal and would never be used by the cultivators for lancing. These results differ from those of Wayne Arny [1917] who working on belladonna found that small plants produced the highest percentage of atropine.

## XI. The Influence of Heredity.

An account of this will appear elsewhere written in conjunction with Mr Leake. The latex produced by more than 500 pure races isolated by him and of crosses made by him has been examined for morphine content and in many cases for codeine content. The pure races represent practically all types of poppy grown in India including white as well as coloured flowered races. Opium from the first lancings of terminal capsules only varied in its morphine content from 6·6 to 20·1 %, reckoned on the dry material. Most of them gave over 11·5 % morphine. The variation in codeine content is from 1·8 to 4·8 %. Indications have been obtained that a race of poppy producing latex of high morphine concentration maintains that power in subsequent seasons.

## XII. The Bearing of the Results on the Function of Alkaloids in Plants.

For the complete literature on the subject the reader must be referred to our main publications.

Our work has established the following facts:

1. When a capsule is lanced for the first time the concentration of morphine in the latex is at a maximum in the first latex to flow out. As the flow continues the morphine concentration diminishes.

2. At each successive lancing the morphine content of the opium obtained decreases rapidly and if sufficient successive incisions are made latex can eventually be obtained which contains no morphine as measured by the method of the British Pharmacopoeia. The morphine is not replaced in the latex by codeine or narcotine. The interval of time between each successive lancing whether one or five days has no influence on the rate of fall in morphine content of the opium from each successive lancing. If the interval of time between the first, second and third lancings is only a few hours, the fall in morphine content at each successive lancing is not so rapid.

3. If only small incisions are made at each successive lancing, and therefore only a small amount of latex removed each time, the fall in morphine concentration of the latex at each successive lancing is much less rapid than when larger incisions are made and more latex removed.

Similarly when for any reason, *e.g.* bad weather conditions or the making of smaller incisions than usual, the yield of the first lancing is low, then the falling off in morphine content is not so great at the second and third lancings as it would have been with a larger yield at the first lancing.

4. In very young capsules, say six days old, the latex is less concentrated in morphine. After the capsules have reached the stage at which they feel firm, say 16 days old in the writer's experiments, the morphine content of the dry opium is the same however long the first lancing is delayed after that stage and however much the yield of opium may vary from day to day.

5. Manuring with nitrogenous manures largely increases the yield of latex but the percentage of morphine in the latex is not largely modified. The yield of dry opium for a particular race of poppy is roughly proportional to the weight of the capsule and incidentally to that of the plant itself.

6. Morphine exists as such in the latex inside the plant.

7. Even though the capsules are lanced to exhaustion of morphine the seed shows no sign of deterioration, *e.g.* in germinating power. It is the universal practice in India to lance as long as the capsules yield latex and the writer knows of no evidence that the seed suffers in any way. The seed when ripe contains no alkaloids whereas the ripe capsules contain considerable quantities even after a year's storage.

8. Climate and weather conditions do not affect the morphine content of the latex to any appreciable degree. They may, however, have important effects on the yield of latex.

Starting with the above established facts it therefore appears that morphine is stored in the capsule more than in other parts of the plant.

This agrees with Clautriau's remarks [1889], who found that the alkaloids were most concentrated in the epidermis of the capsule and diminished in amount towards the lower part of the plant. That morphine is most concentrated in the latex of the capsule seems the only explanation of facts 1 and 2 above. This would explain why the latex first flowing from the cut surface is richest in morphine. As the flow continues, the latex in parts of the plant below the capsule, where it is poorer in morphine, has to be drawn on. This theory fits in well with fact 3 also, because if one only takes out very small amounts of latex at each lancing it will naturally take longer before the less concentrated latex below the capsule is drawn on. This may be accounted for by supposing that a few hours is not sufficient to exhaust the latex already in the capsule when only one incision is made. Actual observations show that the flow continues for at least 16 hours from one cut. If then the second cut is applied within a few hours of the first the latex exuding therefrom will be partly latex which was already in the capsule and therefore richer in morphine and which with one day's interval between the lancings would have all been exuded at the first lanced surface.

The plant loses its power to produce morphine about the stage that its capsules become firm to the touch (vide 4). This explains No. 2, *i.e.* that the morphine content of each successive lancing falls off. The flow of latex appears by No. 6 to be proportionate to the size and the weight of the capsule and incidentally to that of the plants. The effect of nitrogenous manures is to increase the size and weight of the capsules. Yet the morphine concentration in the latex remains nearly the same. It would therefore appear that the amount of morphine produced depends on the amount of plant tissue produced. Different races of poppy however produce latex of different morphine concentration. It may be that plants yielding larger amount of latex may produce

a lower morphine content in that latex and *vice versa*. Data are being accumulated to test this point.

It may be that the amount of morphine produced is proportional to growth whatever the race of poppy. The amount of latex vessels may however be a factor varying with race, and hence a capsule with few latex vessels, *i.e.* one which will give a low yield of latex, may produce opium especially rich in morphine.

It would therefore appear that, during the period of active growth of the plant, morphine is being produced at the same rate as the plant tissue. This fact is against the view of Bayliss [1915], that alkaloids are more or less an accidental product of chemical change. The plant no longer produces morphine when the seed begins to ripen, *i.e.* when its period of active growth has ceased. On the other hand the morphine does not seem to diminish (see No. 4).

The plant deposits morphine chiefly in the capsule, and one of the functions of the lactiferous system would seem to be the removal of alkaloids to the capsule. This theory is supported by the mode of growth of the lactiferous tubes in the case of *Euphorbiae* [Pfeffer, 1900], which send ramifications into each new portion of the tissue in which they occur. No. 7 indicates that the morphine is not used to nourish the seeds. Therefore it would seem that morphine is a useless end product of metabolism. The animal organism takes in complex food materials, and excretes its end products of metabolism which are mainly of a simple structure. The plant on the other hand feeds on simple substances and therefore it is not surprising if some of its end products are complex substances, which it finds difficult to excrete. That the amount of the morphine produced bears a definite relation to the amount of plant growth, would seem to support the theory that it is an excretory product, for the amount of excretory product would naturally depend on the amount of plant growth.

The large number of different alkaloids produced by plants is no objection to the theory that alkaloids are excretory products. Rather it is in favour of it, for certain alkaloids are characteristic of certain families and the metabolism of different families is certain to show differences.

A plant producing much oil in its seed might be expected to form different excretory products from plants producing much starch or protein as reserve material.

## Conclusion.

Morphine in the opium poppy is a useless end product of metabolism. The plant having no mechanism for excreting an end product of such complicated structure stores it in places where it can do no harm to its own metabolism, *i.e.* chiefly in the capsule. The lactiferous system would seem to represent a means of removing waste products of metabolism.

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# LVI. DIGESTIBILITY OF GERMINATED BEANS.

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(*Received July 28th, 1920.*)

It is a well known fact that beans contain a comparatively high percentage of protein which is not readily utilised by animals. Recent work by Chick and Hume [1919] has shown that germinated pulses are much richer in vitamines than are the ungerminated seeds. This result suggested the possibility that on germination of a seed changes may occur which affect the digestibility of the protein; experiments were therefore made in order to discover the effect of germination on the digestibility of bean protein.

Haricot, Brown Dutch and Soya Beans were used and they all gave the same results. In the experiments beans were boiled and digested by means of Benger's "Liquor Pancreatin." After regular intervals portions of the digesting mixture were tested by a method adapted from Sörensen's formaldehyde method.

### *Method of Digestion.*

The liquor in which the beans were boiled was poured off and the beans were washed with cold water (1000 cc.) and powdered.

Sodium chloride (0·75 g.) and toluene (10 cc.) were added. Alkali was added until the reaction of the mixture was alkaline to cresol red and acid to phenolphthalein. Benger's pancreatic fluid (25 cc.) was added and the whole made up with water at 37° to a known volume (500 cc. for 50 g. of beans). The flask was shaken well and 50 cc. of the mixture removed for immediate titration. The neck of the flask was plugged with cotton wool and it was placed in a thermostat at 37°. From this digesting mixture portions were removed when required for titration.

### *Method of Titration.*

The portion of the digesting mixture to be tested was filtered. Five drops phenolphthalein were added to 10 cc. of the filtrate, which was then titrated against dilute NaOH till only very faint pink.

To this 5 cc. of a 40 % solution of formaldehyde (containing one drop phenolphthalein and dilute NaOH till faint pink) were added. The resulting mixture was acid and was titrated against $N/5$ NaOH. This titration was

noted; since the excess of formaldehyde caused any free amino-groups to be neutralised and thus rendered the solution acid, the number of cc. in the last titration gave a measure of the amount of free amino-acids formed by hydrolysis of the protein by the enzyme.

In calculating the amount of nitrogen digested it is assumed that 1 cc. of $N/5$ NaOH used in the titration in presence of formaldehyde corresponds with 0·014/5 g. of N.

*Calculation of Percentage Increase in Digestibility.*

Average nitrogen digested at end of 24 hrs.

(*a*) Ungerminated beans (29 expts) 34·6 mg.

(*b*) Germinated beans (27 expts) 61·7 mg.

(*c*) Germinated and dried beans (20 expts) 49·1 mg.

Average increase obtained by germinating = 27·1 mg. N. This is from 100 cc. bean solution. Increase from 1000 cc. bean solution, which includes 100 g. beans = 271 mg. N.

By Kjeldahl experiments it was shown that 100 g. beans contain 3·18 g. N, so that the percentage increase in digestibility due to germination is 8·5.

Similarly the average increase by germination followed by drying is 4·6 %.

Preliminary experiments were made in order to ascertain:

(1) The correct degree of alkalinity for digestion by pancreatic extract.

(2) The most suitable length of time for boiling the beans.

(3) The effect of boiling NaOH and fat with beans on the digestibility of bean protein. (In both cases the increase obtained was negligible.)

*Results.*

As a result of 29 experiments the average increase in nitrogen digestibility, as indicated by resistance to tryptic activity, of germinated over ungerminated beans was 8·5 %.

The increase varied from 15·3%, when the beans were germinated rapidly, to 4·3 %, when only slow growth took place.

The increase in nitrogen digestibility of dried germinated beans over ungerminated beans varied from 7·8 % to 1·2 % according to the rapidity with which drying occurred. The best method of drying appeared to be to expose the beans to heat such as strong sunlight for one day and then to keep them at a temperature of 30°.

In all cases when the germinated beans were even partially dried, their protein became more resistant to tryptic digestion.

From these experiments it is clear that when beans are germinated their protein is rendered appreciably more digestible—on drying, the protein becomes again less digestible.

*Tabulated Results.*

| No. of Expt. | *Beans* (50 g.) *Series* 1 | Milligrams of Nitrogen digested after (1) 0 hrs. | (2) 24 hrs. | (3) 48 hrs. | (4) 72 hrs. |
|---|---|---|---|---|---|
| (1) | Germinated in saturated air for 24 hrs. at 34° ... ... ... ... | 5·9 | 80·1 | 96·6 | 101·9 |
| (2) | Germinated similarly to (1) ... ... | 7·0 | 75·0 | 95·3 | 101·2 |
| (3) | Ungerminated ... ... ... ... | 6·4 | 47·9 | 66·1 | 71·9 |
| (4) | Ungerminated ... ... ... ... | 6·4 | 45·1 | 64·9 | 71·1 |
| | *Series* 2 | | | | |
| (1) | Germinated in saturated air for 48 hrs. at 34° ... ... ... ... | 6·7 | 76·9 | 110·6 | 130·0 |
| (2) | Germinated similarly to (1) ... ... | 7·6 | 90·0 | 114·3 | 127·6 |
| (3) | Ungerminated ... ... ... ... | 6·1 | 56·0 | 79·3 | 98·0 |
| (4) | Ungerminated ... ... ... ... | 5·6 | 59·8 | 84·0 | 98·0 |
| | *Series* 3 | | | | |
| (1) | Germinated: dried in hot sun for 1 day then at 30° for 4 weeks ... ... | 7·0 | 70·0 | 82·0 | 88·9 |
| (2) | Germinated: dried similarly to (1) ... | 8·1 | 69·2 | 84·3 | 92·0 |
| (3) | Ungerminated ... ... ... ... | 5·8 | 52·1 | 56·2 | 70·3 |
| (4) | Ungerminated ... ... ... ... | 5·8 | 50·1 | 62·1 | 67·4 |
| | *Series* 4 | | | | |
| (1) | Germinated in open air in warm weather for 3 days (radicles 1″ long) ... | 3·9 | 57·1 | 80·0 | 95·9 |
| (2) | Ungerminated ... ... ... ... | 2·0 | 31·9 | 52·1 | 61·8 |
| (3) | Germinated similarly to (1) ... ... | 3·9 | 63·8 | 84·3 | 89·5 |
| (4) | Ungerminated ... ... ... ... | 3·1 | 36·1 | 54·1 | 63·8 |
| | *Series* 5 | | | | |
| (1) | Germinated in greenhouse (30° in day-time) for 3 days ... ... ... | 5·0 | 58·5 | 74·2 | 82·9 |
| (2) | Germinated similarly to (1) ... ... | 5·0 | 48·2 | 70·0 | 80·1 |
| (3) | Ungerminated ... ... ... ... | 3·4 | 36·1 | 47·9 | 52·1 |
| (4) | Ungerminated ... ... ... ... | 3·1 | 32·2 | 45·4 | 47·9 |
| | *Series* 6 | | | | |
| (1) | Germinated in open air for 3 days in warm weather ... ... ... | 7·0 | 45·6 | 65·0 | 75·9 |
| (2) | Germinated similarly to (1) ... ... | 5·9 | 40·0 | 58·5 | 73·9 |
| (3) | Ungerminated ... ... ... ... | 2·0 | 28·6 | 47·9 | 61·9 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 24·1 | 31·4 | 44·0 |
| | *Series* 7 | | | | |
| (1) | Germinated for 2 days: dried in hot sun for 1 day, then at 30° for 6 weeks | 3·1 | 54·0 | 66·1 | 72·5 |
| (2) | Germinated: dried similarly to (1) ... | 3·1 | 47·9 | 61·3 | 68·0 |
| (3) | Ungerminated ... ... ... ... | 2·0 | 28·3 | 42·0 | 49·8 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 30·8 | 42·6 | 47·9 |
| | *Series* 8 | (1) 0 hrs. | (2) 12 hrs. | (3) 24 hrs. | (4) 48 hrs. |
| (1) | Germinated 2 days: dried for 6 weeks at 30° ... ... ... ... | 3·1 | 23·0 | 30·8 | 33·9 |
| (2) | Germinated 2 days: dried for 4 weeks at 30° ... ... ... ... | 3·4 | 21·0 | 29·1 | 36·1 |
| (3) | Ungerminated ... ... ... ... | 2·0 | 10·9 | 17·6 | 24·9 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 9·0 | 16·5 | 23·0 |

*Tabulated Results (continued.*

| No. of Expt. | | Milligrams of Nitrogen digested after (1) 0 hrs. | (2) 12 hrs. | (3) 24 hrs. | (4) 48 hrs. |
|---|---|---|---|---|---|
| | *Series* 9 | | | | |
| (1) | Germinated in open air in very warm weather for 2 days ... ... ... | 5·9 | 31·9 | 45·9 | 61·9 |
| (2) | Germinated similarly to (1) ... ... | 5·0 | 30·8 | 42·0 | 60·4 |
| (3) | Ungerminated ... ... ... ... | 3·1 | 12·9 | 22·1 | 31·1 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 17·1 | 23·0 | 29·1 |
| | *Series* 10 | | | | |
| (1) | Germinated in greenhouse for 2 days at 24° in daytime ... ... ... | 5·0 | 31·1 | 37·0 | 42·0 |
| (2) | Germinated similarly to (1) ... ... | 5·9 | 30·8 | 36·1 | 43·1 |
| (3) | Germinated similarly to (1): dried in room (15° in daytime) for 4 weeks | 3·0 | 17·9 | 26·0 | 33·0 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 15·1 | 22·1 | 26·9 |
| | *Series* 11 | | | | |
| (1) | Germinated in greenhouse for 2 days at 26° in daytime ... ... ... | 5·0 | 40·0 | 47·0 | 52·6 |
| (2) | Germinated similarly to (1) ... ... | 3·9 | 35·0 | 46·5 | 49·0 |
| (3) | Ungerminated ... ... ... ... | 2·5 | 21·0 | 26·0 | 28·3 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 18·8 | 23·0 | 26·6 |
| | *Series* 12 | | | | |
| (1) | Germinated in saturated air at 22° for 2 days ... ... ... ... | 5·9 | 52·1 | 63·9 | 70·0 |
| (2) | Germinated in saturated air at 20° for 2 days ... ... ... ... | 5·0 | 34·2 | 56·0 | 73·9 |
| (3) | Germinated similarly to (1): dried at 30° for 4 weeks ... ... ... | 3·9 | 28·0 | 42·0 | 62·2 |
| (4) | Ungerminated ... ... ... ... | 2·0 | 22·1 | 30·0 | 36·1 |
| | *Series* 13 | (1) 0 hrs. | (2) 24 hrs. | (3) 48 hrs. | (4) 72 hrs. |
| (1) | Germinated: dipped into boiling water for 2 seconds, dried at 30° for 4 weeks | 6·4 | 41·2 | 50·7 | 56·8 |
| (2) | Germinated: dipped, dried in laboratory (15° in daytime) for 8 weeks ... | 7·0 | 33·0 | 38·1 | 40·9 |
| (3) | Germinated: dried in laboratory (15° in daytime) for 8 weeks ... ... | 7·3 | 29·1 | 33·0 | 35·7 |
| (4) | Ungerminated ... ... ... ... | 4·2 | 26·9 | 31·9 | 35·8 |
| | *Series* 14 | (1) 0 hrs. | (2) 12 hrs. | (3) 24 hrs | (4) 48 hrs. |
| (1) | Germinated: dried in hot sun for 1 day then at 30° for 4 weeks ... ... | 8·4 | 50·1 | 61·3 | 72·0 |
| (2) | Germinated: dipped into boiling water, dried in laboratory for 4 weeks ... | 5·9 | 38·1 | 48·4 | 57·7 |
| (3) | Germinated: dried at 30° for 4 weeks ... | 6·7 | 47·6 | 58·5 | 65·0 |
| (4) | Ungerminated ... ... ... ... | 3·9 | 30·5 | 38·9 | 44·0 |
| | *Series* 15 | | | | |
| (1) | Germinated in laboratory at 15° in daytime for 3 days ... ... ... | 6·4 | 31·9 | 50·1 | 68·3 |
| (2) | Germinated in saturated air for 24 hrs. at 30° ... ... ... ... | 9·0 | 57·7 | 71·1 | 80·6 |
| (3) | Germinated in saturated air for 24 hrs. at 34° ... ... ... ... | 8·4 | 54·0 | 75·3 | 91·6 |
| (4) | Ungerminated ... ... ... ... | 3·9 | 24·1 | 33·6 | 43·4 |

*Tabulated Results (continued)*

| No. of Expt. | | Milligrams of Nitrogen digested after (1) 0 hrs. | (2) 12 hrs. | (3) 24 hrs. | (4) 48 hrs. |
|---|---|---|---|---|---|
| | *Series* 16 | | | | |
| (1) | Germinated: dried in hot sun for 1 day then at 30° for 4 weeks ... ... | 5·9 | 54·0 | 66·4 | 78·1 |
| (2) | Germinated: dipped into boiling water, dried at 30° for 4 weeks ... ... | 3·9 | 38·1 | 47·6 | 58·0 |
| (3) | Germinated: dried at 30° for 4 weeks | 5·3 | 47·9 | 59·4 | 66·1 |
| (4) | Ungerminated ... ... ... ... | 3·9 | 32·2 | 41·7 | 47·6 |
| | *Series* 17 | | | | |
| (1) | Germinated in greenhouse for 2 days (30° in daytime) ... ... ... | 8·4 | 61·9 | 82·6 | 96·0 |
| (2) | Germinated in open air in warm weather in shade for 3 days ... ... | 7·3 | 47·0 | 63·8 | 79·2 |
| (3) | Germinated similarly to (2) ... ... | 8·1 | 52·4 | 69·2 | 77·6 |
| (4) | Ungerminated ... ... ... ... | 4·5 | 35·0 | 44·5 | 50·1 |
| | *Series* 18 | | | | |
| (1) | Germinated by placing in funnels plugged with moist cotton wool. A little damp cotton wool was placed over the beans—kept at 27° for 24 hrs. | 5·9 | 52·4 | 63·8 | 75·0 |
| (2) | Germinated similarly to (1) but for 48 hrs. ... ... ... ... ... | 7·6 | 68·0 | 88·5 | 101·9 |
| (3) | Ungerminated ... ... ... ... | 3·9 | 25·5 | 39·8 | 54·0 |
| (4) | Germinated similarly to (2) ... ... | 7·8 | 69·7 | 82·3 | 90·4 |
| | *Series* 19 | | | | |
| (1) | Germinated: dried in laboratory (at 15° in daytime) for 6 weeks ... ... | 5·9 | 39·8 | 52·1 | 62·4 |
| (2) | Germinated: dried in laboratory (at 15° in daytime) for 6 months ... ... | 5·6 | 39·5 | 54·0 | 58·5 |
| (3) | Germinated: dried in laboratory (at 15° in daytime) for 3 months ... | 7·0 | 51·8 | 62·2 | 64·4 |
| (4) | Ungerminated ... ... ... ... | 3·9 | 28·3 | 38·1 | 44·5 |

I desire to express my thanks to Professor Benson of the Royal Holloway College, who suggested the work, for her continual interest and assistance, and to Professor Hopkins of the Biochemical Laboratory, Cambridge, for his advice and help.

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# LVII. THE EFFECT OF PYRUVATES, ALDEHYDES AND METHYLENE BLUE ON THE FERMENTATION OF GLUCOSE BY YEAST JUICE AND ZYMIN IN PRESENCE OF PHOSPHATE.

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(*Received August 3rd, 1920.*)

Oppenheimer [1915] observed that the fermentation of glucose by maceration extract was greatly stimulated by the addition of a pyruvate or pyruvic acid and that acetaldehyde had a similar but less pronounced effect. The estimations were made by weighing at comparatively long intervals but it is obvious in the case of acetaldehyde, that the stimulation chiefly occurs at the commencement of the fermentation. The results with pyruvates are not always so clear and are apparently complicated by some unconsidered factor since in some cases the total $CO_2$ evolved exceeds that obtainable from the glucose and pyruvate in the normal course of fermentation (e.g. $Br_1$, Table III, p. 240 in which 316 milligrams of $CO_2$ are obtained from 0·4 g. of glucose + 0·04 g. pyruvic acid which would together only yield in the normal course 220 mgms. or including the observed autofermentation 230 mgms., and $Br_2$ in the same table where 295 mgms. are obtained against 212 normally obtainable).

Neuberg somewhat later [1915] observed a similar stimulating action of pyruvates and other $\alpha$-ketoacids on the fermentation of glucose, mannose, fructose and saccharose and remarked that the activation was most pronounced at the commencement of the fermentation. Experiments continued for 19–20 hours (p. 82) showed little difference in the total fermentation in the presence and absence of pyruvate.

Neuberg subsequently examined the effect of a large number of aldehydes [1918] on alcoholic fermentation and found that they were all vigorous activators. He pointed out that the effect was most marked with glucose and mannose, less so with fructose and cane sugar and suggested that this fact might be related to the observation of Harden and Young [1909] that fructose under certain circumstances can stimulate the fermentation of glucose. The stimulation, like that produced by pyruvate, was most marked at the commencement of fermentation.

Meyerhof [1918] has made an interesting contribution to this subject in his study of the kinetics of cell-free fermentation. Lebedev [1912] observed that when maceration extract was mixed with a fermentable sugar a period of *induction* occurred during which no $CO_2$ was formed and no change in rotation occurred, and this has frequently been confirmed. Meyerhof now finds that this induction is not observed when the extract contains even a trace (0·2 millimolecular concentration) of a hexosephosphate and hence has never been recorded for juice prepared by Buchner's process, in which recognisable amounts of hexosephosphate are present. The period is moreover shorter in presence of cane sugar than of fructose or glucose and is diminished when these two sugars are warmed for 4–6 hours at 80° with a neutral phosphate mixture. The induction period is also greatly lessened by grinding the dried yeast with glass powder before maceration.

Following on the induction period, the velocity of fermentation more or less gradually attains a maximum corresponding to the concentration of phosphate present. This is termed by Meyerhof the "Gäranstieg" and has been studied in some detail. The effects of the addition of excess of phosphate described by Harden and Young [1908], viz. a diminished maximum and a more gradual attainment of the maximum[1], are found by Meyerhof to be also produced by the addition of salts such as sodium chloride or nitrate and hence the phenomenon is partly to be explained as a general salt effect. The rate of attainment of the maximum becomes greater as the hexosephosphate concentration increases, but this characteristic phenomenon cannot entirely be abolished by the addition of hexosephosphate. Meyerhof discusses the cause of the phenomenon and shows by an ingenious application of the effect of arsenates that it is probably not due to the production of a specially labile form of sugar from the hexosephosphate. The attainment of the maximum was also found to be more rapid the greater the concentration of the coenzyme, added in the form of boiled extract of yeast or muscle.

The facts that the activating effect both of $\alpha$-ketoacids and of aldehydes was chiefly manifested at the commencement of the reaction, that the experiments both of Oppenheimer and Neuberg were made with maceration extract which contains a large amount of mineral phosphate and that the effect was less marked with fructose than with glucose led us to enquire whether this action was a general stimulation of the fermentation process or a more specific acceleration of the reaction in presence of free mineral phosphate. The results show that when aldehyde is added to fermenting mixtures of yeast juice or

[1] Meyerhof [1918, p. 196] erroneously attributes to Harden and Young in explanation of this phenomenon the suggestion that the *sugar* forms with high and low concentrations of phosphate different esters of different stability, one of which, as the phosphate is used up, passes into the other. What they actually suggested was [Harden and Young 1908] that the phosphate is capable of forming two or more different unstable associations with the *fermenting complex* (by which was meant the complex of enzymes concerned, not the sugar). The alternative suggestion has also been made [see Harden 1914] "that the addition of increasing amounts of phosphate causes a progressive but reversible change in the mode of dispersion of the colloidal enzyme."

zymin (acetone yeast) with glucose no marked acceleration in the normal rate of fermentation occurs. If a suitable amount of phosphate be then added, sufficient to cause only a gradual rise of rate to the maximum in the control experiment with glucose, the effect of the presence of the aldehyde is greatly to diminish the time required for the attainment of the maximum, so that the volume of gas evolved in the period immediately following the addition of the phosphate is greatly increased. At the same time a considerably higher maximum is attained. On the completion of the esterification of the phosphate, the rate again diminishes both in the presence and absence of aldehyde and the total evolution is not greatly different in the two cases. Similar phenomena are produced by the addition of pyruvates. The effect varies with the concentration of aldehyde and is common, but in unequal measure, to the four aldehydes tested (formic, acetic, propionic and butyric). So far only glucose has been employed as the fermentable sugar, but experiments are in progress with fructose and cane sugar.

This striking effect of aldehydes, which are known to be readily reducible by yeast, strongly suggested that the cause of the delay in attainment of the maximum after the addition of phosphate was lack of an acceptor for hydrogen. In order to test this idea, methylene blue, which is also readily reducible by yeast, was substituted for the aldehyde, with the result that it was found to produce a very similar effect. When increasing amounts of methylene blue are added a point is soon reached at which the maximum, although it is more rapidly attained, is considerably lowered. This is probably due to the inhibitory effect of the dye on the enzyme complex. Even with the most favourable concentration of methylene blue however the rise of rate was not so rapid as with the aldehydes, and the maximum was unchanged.

According to the pyruvic acid theory, which may now be taken as established, the final stage of the alcoholic fermentation of sugar is the reduction of acetaldehyde (produced by the decomposition of pyruvic acid), a reaction which proceeds so rapidly that only an extremely small concentration of the aldehyde is present during normal fermentation.

$$CH_3 . CO . COOH \longrightarrow CH_3 . CHO + CO_2 \xrightarrow[+2H]{} CH_3 . CH_2OH + CO_2.$$

Further, the production of the pyruvic acid from sugar appears only to be possible when some acceptor for hydrogen is available, this being normally supplied by the acetaldehyde produced in a later stage of the reaction.

On this view it would seem to follow that no rise in the rate of fermentation can occur without the provision of an additional quantity of a hydrogen acceptor. Some such acceptor is probably more or less rapidly formed and reduced during the period of delay, which follows on the addition of phosphate, this process being accompanied by a corresponding increase in the formation of pyruvic acid, until sufficient of this is being produced to provide the amount of acetaldehyde necessary for the maximum effect. When, however, the easily reducible aldehydes or methylene blue are added, these act as

acceptors and a much more rapid or even instantaneous attainment of the maximum becomes possible, as was actually observed in our experiments.

Whether this acceptor is the same substance as yields glycerol in the sulphite fermentation of Neuberg and Reinfurth, and is supposed by Neuberg to be methylglyoxal, is uncertain. It may be pointed out however that the precursor of glycerol assumed by Neuberg and Kerb [1913] must be much less rapidly reduced than acetaldehyde in the course of normal alcoholic fermentation since the ratio of glycerol to alcohol under these circumstances is only small. No experiments have yet been made to decide whether an enhanced glycerol production occurs during the period of delay.

It seems probable that the delay following the addition of phosphate when fructose is employed as the fermentable sugar is also due in part to lack of an acceptor. The facts that fructose yields a much higher maximum rate with phosphate and that the optimum concentration of phosphate is much higher than for glucose can accordingly be interpreted to mean either that fructose yields a hydrogen acceptor much more readily than glucose or that the acceptor formed is much more rapidly reducible. This question is at present under investigation. If this conclusion be granted, a simple explanation is afforded of the remarkable "induction" observed by Harden and Young [1909] when fructose was added to a mixture of yeast juice and glucose or mannose to which a considerable excess of phosphate had been added. Under these circumstances the rate of attainment of the maximum fermentation was greatly accelerated even when the phosphate concentration was kept constant and moreover the volume of $CO_2$ evolved under these circumstances was much greater than could be obtained from the fructose added. In the light of the foregoing remarks it now appears that the function of the fructose under these conditions is probably to provide a hydrogen acceptor and this, once formed, enables the fermentation of the glucose to proceed rapidly, as explained above, even in the presence of a concentration of phosphate, which in the absence of an acceptor causes a prolonged delay.

It is further probable that the hydrolysis of the hexosephosphate, both that originally present and that slowly formed in the fermenting mixture, results in the formation of fructose, which in its turn yields a hydrogen acceptor and thus assists in the increase of the rate of fermentation. Meyerhof's observations on the marked effect of hexosephosphate on the rate of attainment of the maximum would thus receive a simple explanation.

Owing to the method of experiment employed by us, the full effect of the addition of hexosephosphate could not be observed as the fermenting mixtures always contained this substance formed from the phosphate originally present. The addition of a further quantity of hexosephosphate produced very little effect.

Whether the lack of acceptor combined with Meyerhof's "salt-effect" of the excess of phosphate provides a complete explanation of the delay in

attainment of the maximum after the addition of phosphate or whether time is required for some other change, such as transformation of the sugar into a fermentable form, as maintained by Euler, remains as a subject for further investigation.

## Experimental.

The yeast juice and zymin employed were both prepared from a brewery top-yeast. The acetaldehyde used in Exp. 2 was a preparation obtained from Kahlbaum. For the other experiments it was prepared from paraldehyde by distillation with dilute sulphuric acid. The formaldehyde was a dilution of formalin and the concentration was estimated by Ripper's method. The propionic aldehyde was prepared by heating a mixture of calcium propionate and formate and the butyric aldehyde by the oxidation of *n*-butyl alcohol with potassium dichromate and sulphuric acid. The methylene blue was Grübler's "Methylenblau med pur." The pyruvate solution was made by dissolving 1 g. of pyruvic acid in water, neutralising with *N* KHO and making to 100 cc.

*Effect of the addition of pyruvate and acetaldehyde to yeast juice in presence and absence of free phosphate.*

*Exp.* 1. 25 cc. Yeast-juice + 1 g. glucose. No toluene. T = 25°.

| | 1% Pyruvate cc. | 1% Acetaldehyde cc. | $H_2O$ cc. | | 0·3*M* $Na_2HPO_4$ cc. | $H_2O$ cc. |
|---|---|---|---|---|---|---|
| 1 | 5 | 0 | 0 | and subsequently | 5 | 0 |
| 2 | 5 | 0 | 0 | | 0 | 5 |
| 3 | 0 | 5 | 0 | | 5 | 0 |
| 4 | 0 | 5 | 0 | | 0 | 5 |
| 5 | 0 | 0 | 5 | | 5 | 0 |
| 6 | 0 | 0 | 5 | | 0 | 5 |

Measurements were commenced at 2.20. The additions of phosphate and water were made at 3.20.

| | Pyruvate | | Aldehyde | | Water | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| cc. $CO_2$ evolved before addition of phosphate in 55′ | 23·6 | 23·8 | 20·9 | 21·2 | 22·3 | 23·2 |
| cc. $CO_2$ evolved after addition of phosphate in successive periods of 5′ | | | | | | |
| | $Na_2HPO_4$ | $H_2O$ | $Na_2HPO_4$ | $H_2O$ | $Na_2HPO_4$ | $H_2O$ |
| 1 | 17·8 | 1·9 | 29·8 | 2 | 5·2 | 1 |
| 2 | 24·0 | 2 | 12·8 | 2 | 7·8 | 3 |
| 3, 4 | 6·7 | 4·6 | 7·4 | 3·7 | 28·1 | 4·2 |
| 5, 6 | 4·7 | 3·4 | 1·2 | 3·4 | 7·3 | 3·7 |
| Total in 30′ | 53·2 | 11·9 | 51·2 | 11·1 | 48·4 | 11·9 |

It will be seen that the addition of pyruvate or aldehyde did not appreciably alter the normal rate of fermentation, as in the 55′ preceding the addition of phosphate all six flasks gave approximately the same amounts of gas. Further,

the three flasks 2, 4 and 6 to which $H_2O$ was subsequently added continued to ferment at equal rates, giving almost exactly equal amounts of gas in 30′ (11·9, 11·1 and 11·9 cc.).

On the addition of phosphate, flask 6, containing no aldehyde or pyruvate, showed the normal behaviour, the rate gradually rising to a maximum which was attained in 10–15′ and amounted to 15 per 5′ (calculated by plotting the figures given in the table). In presence of aldehyde the rate rose to its maximum of 29·8 in the first 5′, whilst in presence of pyruvate an intermediate result was obtained the evolution being 17·8 in the first 5′ and 24 in the second.

*Exp.* 2. 25 cc. Yeast-juice + 1 g. glucose. No Toluene. T = 25°.

| | 1% Pyruvate cc. | Water cc. | | 0·3*M* $Na_2HPO_4$ cc. | $H_2O$ cc. |
|---|---|---|---|---|---|
| 1 | 5 | 0 | and subsequently | 5 | 0 |
| 2 | 5 | 0 | | 0 | 5 |
| 3 | 0 | 5 | | 5 | 0 |
| 4 | 0 | 5 | | 0 | 5 |

Measurements were commenced at 3.10 and the additions of phosphate and water were made at 3.40.

| | Pyruvate | | Water | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| cc. $CO_2$ evolved before addition of phosphate between 3.10 and 3.30. | 6·9 | 7·1 | 7·2 | 7 |

cc. $CO_2$ evolved after addition of phosphate in successive periods of 5′.

| | Phosphate | Water | Phosphate | Water |
|---|---|---|---|---|
| 1 | 3·4 | 0·3 | 0·3 | 0·5 |
| 2 | 5·6 | 0·5 | 1 | 0·7 |
| 3 | 4·4 | 0·4 | 0·8 | 0·4 |
| 4, 5 | 10·2 | 0·8 | 1·6 | 0·8 |
| Total in 25′ | 23·6 | 2 | 3·7 | 2·4 |
| Total in 140′ | 49 | 9·7 | 31·2 | 9·2 |
| Total in 17 hrs. | 88·6 | 49·5 | 76·2 | 46·1 |

This is an example in which a quantity of phosphate largely in excess of the optimum was added. In the absence of added phosphate (Nos. 2 and 4) the fermentation was substantially the same with and without pyruvate. After addition of phosphate in the presence of pyruvate the rate rose much more rapidly than in its absence and in the latter case, probably owing to the continued action of excess of phosphate on the enzyme complex, the total was considerably less.

*Effect of varying concentrations of acetaldehyde on the fermentation of glucose by zymin in presence and absence of phosphate.*

*Exp.* 3. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0·2 cc. Toluene. Acetaldehyde 1% solution (by weight). T = 25·8°.

| | 1% Acetaldehyde cc. | |
|---|---|---|
| 1 | 0 | } and subsequently 10 cc. 0·15*M* $Na_2HPO_4$ to each. |
| 2 | 1·2 | |
| 3 | 6 | |
| 4 | 12 | |

Incubated for 1 hour and the phosphate then added.

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | | Acetaldehyde | | |
| | 0 cc. | 1·2 cc. | 6 cc. | 12 cc. |
| cc. $CO_2$ evolved before addition of phosphate in 45′. | 34·4 | 33·6 | 20·7 | 3·4 |
| cc. $CO_2$ evolved after addition in successive periods of 5′. | | | | |
| 1 | 2·7 | 3·4 | 12·5 | 5·5 |
| 2 | 3·6 | 4·6 | 12·8 | 8·4 |
| 3 | 5·3 | 5 | 12·3 | 9·7 |
| 4 | 6·2 | 7·2 | 11·7 | 9·4 |
| 5 | 7·7 | 7·7 | 9·8 | 9·0 |
| 6 | 9·1 | 9·6 | 9·3 | 7·9 |
| 7 | 9·0 | 9·3 | 7·2 | 7·5 |
| 8 | 8·0 | 7·5 | 3·6 | 7·4 |
| Total in 40′ | 51·6 | 54·4 | 79·1 | 64·8 |
| Total in 110′ | 113·6 | 118·8 | 119·6 | 103·1 |

The characteristic effect is here well marked. In the control (No. 1) the maximum of 9·1 cc. is slowly reached in 30–35′ and the rate then as gradually diminishes. 1·2 cc. of 1% aldehyde [1 in 1667] produce practically no effect, 6 cc. on the other hand [1 in 334] produce a very marked effect, the maximum is much higher (12·8 cc.) and is very rapidly attained (5–10′). The total evolved in 110′ is however only about 5% greater than that of the control. A point of considerable interest is that both 6 cc. and 12 cc. of 1% aldehyde diminish the normal rate of fermentation although they both produce a considerable acceleration in the rate at which the maximum is attained after the addition of phosphate. This is especially marked in the case of the 12 cc. of aldehyde solution, which diminished the normal rate of fermentation to 1/10 of that of the control. This effect is probably due to a specific inhibition of the hexosephosphatase, thus diminishing the liberation of phosphate on which the normal rate depends.

*Effect of formic, acetic, propionic and butyric aldehydes on the fermentation of glucose by yeast-juice in presence of phosphate.*

*Exp.* 4. 25 cc. Yeast-juice + 1 g. Glucose + 0·2 cc. Toluene. T = 25°.

| | | |
|---|---|---|
| 1 | 5 cc. Water | } and then 10 cc. 0·3*M* $K_2HPO_4$. |
| 2 | „ 0·78% Formaldehyde | |
| 3 | „ 1% Acetaldehyde | |
| 4 | „ 1·3% Propionic Aldehyde | |
| 5 | „ 1·6% Butyric Aldehyde | |

Incubated for 50′ before addition of phosphate.

| | 1 Water | 2 Formaldehyde | 3 Acetaldehyde | 4 Propionic Aldehyde | 5 Butyric Aldehyde |
|---|---|---|---|---|---|
| cc. $CO_2$ evolved in 30′ before addition of phosphate | 7·3 | 4·1 | 7·0 | 7·7 | 7·2 |
| cc. $CO_2$ evolved after addition of phosphate in successive periods of 5′. | | | | | |
| 1, 2 | 15·5 | 23·6 | 57·1 | 58·1 | 53·7 |
| 3 | 12·5 | 22·4 | 16·4 | 16·6 | 16·1 |
| 4 | 15·3 | 20·7 | 2·7 | 2·7 | 2·8 |
| 5 | 18·4 | 7·0 | 1·6 | 1·5 | 1·7 |
| 6 | 11·9 | 1·4 | 1·2 | 1·6 | 1·4 |
| 7 | 2·5 | 1·5 | 1·8 | 1·6 | 1·4 |
| | 76·1 | 76·6 | 80·8 | 82·1 | 77·1 |

*Exp.* 5. As above, but 15 cc. of 0·3*M* $K_2HPO_4$ added. Incubated 25′.

| | 6 Water | 7 Formaldehyde | 8 Acetaldehyde | 9 Propionic Aldehyde | 10 Butyric Aldehyde |
|---|---|---|---|---|---|
| cc. $CO_2$ evolved after addition of phosphate in successive periods of 5′. | | | | | |
| 1, 2 (10′) | 10·3 | 22·9 | 56·8 (28·4, 28·4) | 55·0 (26, 29) | 48·1 (23, 25·1) |
| 3 | 5·5 | 22·3 | 27·6 | 27·2 | 30·1 |
| 4 | 6·6 | 23·9 | 20·5 | 19·9 | 22·4 |
| 5 | 8·0 | 21·1 | 4·8 | 7·9 | 3·8 |
| 6 | 10·3 | 15·7 | 1·6 | 2·1 | 1·8 |
| 7 | 11·3 | 2·4 | 1·3 | 1·6 | 1·5 |
| 8 | 15·3 | 1·4 | 1·4 | 1·4 | 1·2 |
| 9 | 17·8 | | | | |
| 10 | 13·9 | | | | |
| 11, 12 | 4·5 | | | | |

With this sample of juice very high maxima were attained, but as the first readings were made 10′ after the addition of phosphate the exact maximum could not be directly observed. They were obtained by plotting the total fermentations and reading the evolutions per 5′ from the curves, with the following approximate results.

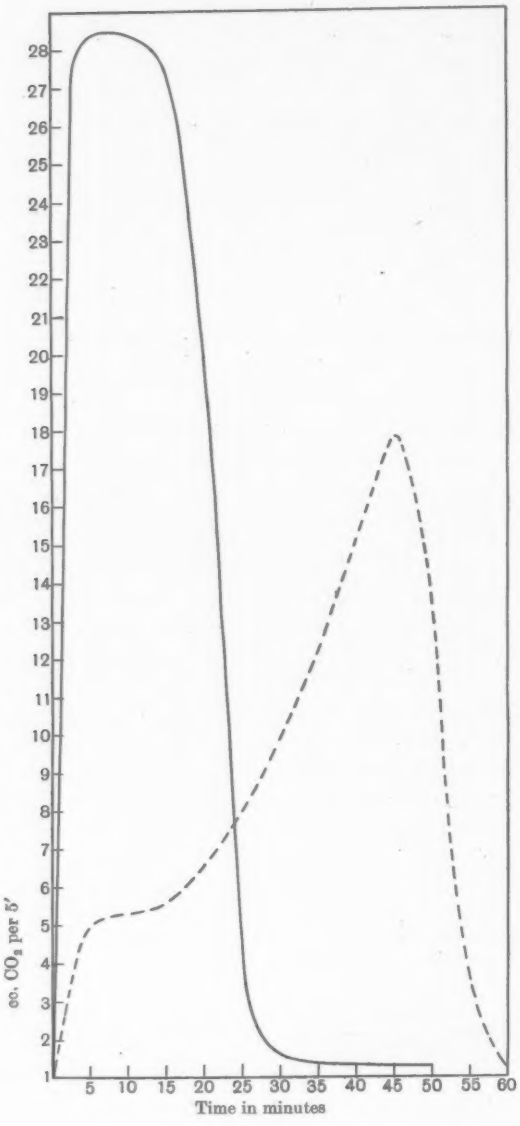
*Exp.* 4.

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Maximum ... ... | 18·4 | 22·4 | 34 | 35 | 27 |
| Minutes after addition when attained ... | 20–25 | 30–15 | 0–5 | 0–5 | 0–5 |

*Exp.* 5.

| | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|
| Maximum ... ... | 17·8 | 23·9 | 25·4 | 29 | 30·1 |
| Minutes after addition when attained ... | 40–45 | 15–20 | 0–5 | 5–10 | 30–15 |

The effect here is very marked and moreover in several cases the maximum is reached in the first 5′ of fermentation. The results with the acetaldehyde in No. 8, Exp. 5, are plotted in Fig. 1.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4
3
2
1
cc. $CO_2$ per 5′
5 10 15 20 25 30 35 40 45 50 55 60
Time in minutes

Fig. 1.

*Effect of (a) Acetaldehyde, (b) Na Hexosephosphate on the fermentation of glucose by zymin in presence of phosphate.*

*Exp.* 6. 4 g. Zymin + 2 g. Glucose in 20 cc. + 0·2 cc. Toluene. T = 25·8°.

1% Acetaldehyde

| | | | |
|---|---|---|---|
| 1 | 0 | 1 | 10 cc. 0·3*M* $K_2HPO_4$ |
| 2 | 1·2 cc. | 2 | |
| 3 | 6 cc. | 3 | |
| 4 | 0 | 4 | 10 cc. $H_2O$ + 10 cc. 0·3*M* $K_2HPO_4$ |
| 5 | 0 | 5 | Na Hexosephosphate from 0·2 g. Basalt + 0·52 g. $K_2HPO_4$ in 20 cc. (equivalent to 10 cc. 0·02*M* Hexosephosphate and 10 cc. 0·3*M* $K_2HPO_4$). |

(1, 2, 3, 4, 5: and subsequently)

Incubated for 65′ before the additions were made.

| | Water 1 | 1·2 cc. Acetaldehyde 2 | 6 cc. Acetaldehyde 3 | 4 | 5 |
|---|---|---|---|---|---|
| cc. $CO_2$ evolved before additions in 50′ | 39·2 | 33·8 | 28 | 38·9 | 38·4 |

Additions made at 1·05.

cc. $CO_2$ evolved after addition in successive periods of 5′.

| | Phosphate | Phosphate | Phosphate | Phosphate and water | Phosphate + Hexose-phosphate |
|---|---|---|---|---|---|
| 1 | 2·7 | 3·2 | 13·6 | 0·3 | 3·5 |
| 2 | 2·9 | 2·7 | 15·6 | 1·8 | 3·8 |
| 3 | 4·3 | 4·1 | 15·4 | 3·0 | 4·4 |
| 4 | 5·0 | 5·4 | 15·6 | 4·3 | 6·1 |
| 5 | 7·3 | 7·7 | 13·4 | 5·0 | 15·6 |
| 6 | 9·1 | 9·5 | 12·1 | 8·1 | |
| 7 | 10·5 | 10·7 | 11·1 | 9·2 | 8·8 |
| 8 | 11·4 | 12·3 | 10·0 | 10·3 | 8·8 |
| 9 | 11·0 | 10·4 | 8·0 | 10·3 | 8·6 |
| 10 | 10·9 | 10·8 | 6·4 | 9·2 | 8·0 |
| 11 | 9·6 | 9·1 | 3·6 | 8·9 | 14·6 |
| 12 | 8·4 | 8·1 | 3·4 | 8·6 | |
| Total in 60′ | 93·1 | 94 | 128·2 | 79·0 | 82·4 |
| Total in 120′ | 157 | 169·2 | 169·3 | 152·3 | 144·9 |

(*a*) The result with acetaldehyde confirms that obtained in Exp. 3, but the total in 2 hrs. is slightly larger in presence of acetaldehyde than in its absence.

(*b*) The addition of hexosephosphate has only a small effect in accelerating the attainment of the maximum. The total in 2 hours is slightly less than that in the control.

*Effect of varying concentrations of methylene blue on the fermentation of glucose by zymin in the presence of phosphate.*

*Exp.* 7. 4 g. Zymin + 2 g. Glucose in 20 cc. 0·2 cc. Toluene. T = 25·5°.

| | | |
|---|---|---|
| 1 | Methylene Blue 0 | and subsequently 10 cc. 0·3*M* $K_2HPO_4$ to each. |
| 2 | „ 0·1 g. | |
| 3 | „ 0·2 g. | |
| 4 | „ 0·3 g. | |

The results of 1 and 2 are shown in the curves (Fig. 2) in which *A* and *B* represent the evolutions per 5′ in absence and presence of methylene blue respectively.

In presence of 0·2 g. and 0·3 g. of methylene blue the course of the reaction was almost the same as with 0·1 g. but the maximum attained was in each case slightly lower (0·1 g., 13·2 cc.; 0·2 g., 12·5; 0·3 g., 12·7). The methylene blue in (2) became colourless in about 1 hr. and in (3) in about 2 hours.

The totals evolved in 2hr. 15 min. in the 4 flasks were almost identical, as will be seen from the following statement.

| No. | M.B. g. | Max. attained (cc. in 5′) | Total in 2 hrs. 15 min. cc. |
|---|---|---|---|
| 1 | 0·0 | 13·2 | 181·2 |
| 2 | 0·1 | 13·2 | 184·5 |
| 3 | 0·2 | 12·5 | 176·8 |
| 4 | 0·3 | 12·7 | 178·9 |

When a still larger concentration of methylene blue is employed, the maximum is considerably lower than in its absence but is more quickly attained. The dye apparently partially inhibits the enzyme complex.

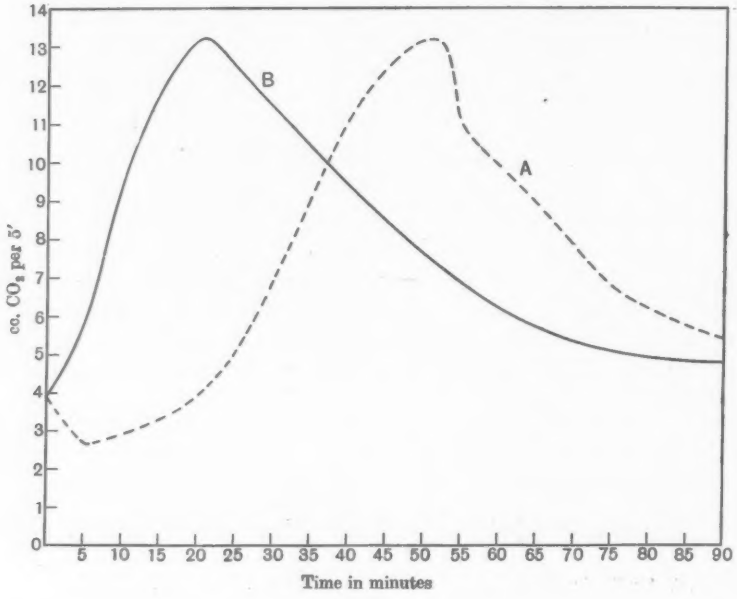


Fig. 2.

*Exp.* 8. 4 g. Zymin + 2 g. Glucose in 20 cc. +0·2 cc. Toluene. T=26°.

| | | | |
|---|---|---|---|
| 1 | Methylene Blue 0 | | |
| 2 | ,, 0·5 g. | and subsequently | 6 cc. 0·3*M* $K_2HPO_4$ |
| 3 | ,, 0 | | |
| 4 | ,, 0·5 g. | | 10 cc. 0·3*M* $K_2HPO_4$ |

*Result.* After the addition of phosphate.

| | Phosphate added 0·3*M* | M.B. g. | Max. attained | Time required to attain maximum | Total evolved in 1 hr. 35 min. |
|---|---|---|---|---|---|
| 1 | 6 | 0 | 11 | 30–35′ | 107·0 |
| 2 | 6 | 0·5 | 8·8 | 0–5′ | 97·4 in 1 hr. 55 min. |
| 3 | 10 | 0 | 12·2 | 30–35′ | 147·1 |
| 4 | 10 | 0·5 | 10·6 | 5–10′ | 149·7 |

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# LVIII. THE DISTRIBUTION OF INORGANIC IRON IN PLANT AND ANIMAL TISSUES.

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## Experimental Methods.

In carrying out tests for the detection of inorganic iron in the tissues, two points must be borne in mind, firstly, the previous preparation of the tissue, and, secondly, the application of the reagents so that false results are not obtained.

With plant tissues when the question is not the structural arrangement but rather whether this or that cell-constituent contains iron, it is better to work with finely teased or broken-up tissues. Glass rods, drawn to a point, were used for this purpose, and also in order to break up some of the green cells and set free the chloroplasts, a portion of the tissue in each case was still more broken up by turning upon it the blunt end of the glass rod and grinding it between this and the microscope slide on which it was being mounted.

In tissues which contained a large quantity of chlorophyll, or where it was desired to stain the stroma of the chloroplast for inorganic iron, the tissues were first boiled in absolute alcohol several times until they became colourless, the greenish extract being poured off with each fresh quantity of alcohol.

In the case of animal tissues, organisms of small size can be mounted direct, but with larger tissues sections have to be cut. The tissues for this purpose were hardened in absolute alcohol for 24 hours, and then embedded in paraffin.

The sections were mounted on slides and after removing the paraffin by xylene, were "taken down" the alcohol-water series before staining.

Preliminary observations were made upon plant tissues, with the ammonium sulphide and ferrocyanide methods.

## Ammonium Sulphide Method.

This method was introduced by Vogel in 1845. The solution used was one containing ammonium hydrogen sulphide, prepared by passing sulphuretted hydrogen through dilute ammonia having a specific gravity of 0·96. The teased tissues were placed in this solution (which must be freshly prepared) for three or four hours, and then mounted in the stain, ringing the coverslip with gold size.

The inorganic iron in the tissues is stained brownish-black, while the rest of the tissues are stained a lighter brown.

The chief disadvantages of the method are: firstly, the difficulty of recognising the iron when present in small quantities, because, the rest of the tissue being stained brown, it is only by very careful comparisons in tissues of equal thickness that the presence of iron can be detected with certainty; and secondly, permanent sections can only be made with difficulty.

### Ferrocyanide Method.

This method was first used by Perls [1879] and ater by Molisch [1893], who was the first to differentiate between inorganic and "masked" iron.

The tissues are placed in a freshly prepared solution of 1·5 % potassium ferrocyanide and a 5 % solution of hydrochloric acid, for three or four hours, and then mounted in the stain and the coverslip ringed with gold size.

It is advisable to use ferrocyanide solution rather than ferricyanide, as the iron is chiefly present in the ferric form, and consequently then gives "Prussian blue" at once. When treated in this way the tissues tend to vary in their depth of staining, this becoming more intense during the first few days, especially if exposed to day-light, and then gradually fading to a much lighter colour.

Another disadvantage of this method is, that if permanent sections are made in Canada balsam, they fade rapidly, this being probably due to the reduction of the stain by means of the balsam forming a compound analogous to $Fe_2Fe(CN)_6$, which is white in the absence of oxygen [Mann, 1902].

### Haematoxylin Method.

This method, which was introduced by Macallum [1897], depends on the fact that when a solution of haematoxylin in pure distilled water is mixed with a very dilute solution of an ordinary iron salt, such as ferric chloride, a deep blue-black coloration is immediately produced.

If, instead of a solution of an ordinary iron salt, a solution of highly colloidal iron or dialysed iron hydroxide be mixed with the stain, a brownish colour is produced, while if organic iron is used, no change in colour takes place. This fact has been explained by Roscoe and Schorlemmer as due to the organic iron compounds having all their possible valences united to carbon atoms.

This staining as a test for iron is quite different from the ordinary use of haematoxylin as a nuclear stain, in which case a mordant is always used, either preceding the haematoxylin, as for example, the iron-alum mordant in Heidenhain's haematoxylin-iron method, or simultaneously, as in the use of the haem-alum stain.

In Macallum's process no mordant whatever is used, but only a solution of haematoxylin in pure distilled water. This gives the colour change only where

a mordant, *e.g.* iron, is naturally present in the tissues. In using it not only must even minutest traces of iron in the water and other fluids be avoided, but also all traces of alkali and acid, since these interfere with the delicacy of the reaction—alkali gives a rose-red colour with the haematoxylin, and acid inhibits the development of the blue-black colour when the amount of iron is small.

To make the staining solution 0·3 gram of pure haematoxylin is dissolved in 50 c.c. of twice distilled water, and kept in Jena glass flasks, since the alkali dissolved out from ordinary glass rapidly turns the solution pink.

Plant tissues, after being prepared, were stained for 12 hours in this solution, while sections of animal tissues were stained for 48 hours.

This was found to be by far the most satisfactory method for the detection of the inorganic iron, and was used in all the animal tissues, and nearly all the plant tissues investigated. Sections so stained can readily be made permanent by dehydrating with alcohol, and mounting in Canada balsam.

## Experimental Results.

### *Section A. Plant Tissues.*

In examining plant tissues, it was found that taken as a whole they gave the iron reactions more rapidly and intensely than animal tissues.

In the various grades of plant life it was found that the lower organisms gave a more intense reaction than those higher in the scale. The staining occurred in three different places—in the nuclei, in the chloroplasts, and in large masses scattered throughout the cytoplasm. Leaves of the wall-flower did not show any definite iron staining. Leaves of ordinary water-cress, gave a very definite reaction for iron; large masses, stained bluish-black, were scattered irregularly throughout the leaves, and many of the chloroplasts were also stained.

Descending lower in the scale, sections of various types of sea-weed were examined. The majority of these showed definite iron staining, particularly in the nuclei and the chloroplasts. The staining in the nuclei was specially well marked, while in some of the sections there were also large deeply-stained masses irregularly situated in the cytoplasm.

The reactions for inorganic iron were most marked with the lower plants, such as unicellular green plants, isolated or bound together in delicate alga threads. The algae observed were Vaucheria, Spirogyra Ulva, and Ulothrix. The nuclei and chloroplasts took on a very deep bluish-black colour very readily; the rest of the cytoplasm was usually unstained, but sometimes patches giving the brown colour of colloidal iron were observed.

Diatoms readily took on a dark blue colour, the central part of the cell more intensely than the rest.

*Section B. Animal Tissues.*

In this series the maximum amount of staining occurred in the lower organisms, *e.g.* Crustacean and Molluscan types, while the Mammalia gave only slight reactions.

In adult *Mammalian tissues* taken from guinea-pigs, sections of the liver showed only very slight iron-staining. This occurred chiefly in the nuclei of the cells in the form of minute, darkly-stained granules, readily visible with the $\frac{1}{12}''$ objective.

In sections of the stomach, the epithelial cytoplasm was unstained, but the granules in the nuclei, specially the nuclei of the muscle fibres, were well stained.

The spleen shows very definite staining, in the form of small irregular granules scattered in large numbers throughout the tissue; granules in the nuclei are also stained.

Sections of the kidney, testicle, and ovary show only the granular staining in the nuclei, except that in the kidney there is also some slight staining of the glomeruli.

In all the mammalian tissues the staining is more marked in the cells which immediately surround the blood vessels.

In *foetal tissues*, taken from the guinea-pig, the reaction was in most of them much more readily obtained than in adult tissues.

In the foetal liver, both the granules scattered throughout the cytoplasm and those in the nuclei were more deeply stained than in the adult liver.

In the foetal kidney the staining was similar to that found in the adult.

The spleen gave very definite reactions, both the granules in the nuclei and the granules irregularly scattered throughout the cytoplasm being more deeply stained than in adult tissues.

*Placental tissues*, also taken from the guinea-pig, showed very definite staining in patches in the chorionic villi, while most of the nuclei were also stained.

In *human blood smears* only a slight blue coloration of the red cells took place, not sufficient to indicate the presence of inorganic iron.

In *avian tissues*, taken from the sparrow, inorganic iron could be very readily detected. In the liver the sections showed large numbers of darkly stained granules scattered throughout the cytoplasm and also in the nuclei. The cytoplasm (when using a high magnification) also shows very minute smaller granules which give an intense reaction for inorganic iron.

In the kidney, the glomeruli and the lumina of the convoluted tubules are well stained.

In the spleen, no staining could be detected, and in the ovary staining was confined to the nuclei.

These avian tissues on the whole, specially the liver, gave a much more definite reaction for iron than did the mammalian tissues.

In *amphibian tissues*, taken from the frog, the reactions were still more marked. In the liver, there were large numbers of small darkly-stained granules, scattered throughout the cytoplasm, in addition to the well-known pigment masses which occur in unstained sections of frog's liver.

In the kidney, the chief staining took place in the granules of the nuclei.

The spleen showed small darkly-stained granules scattered throughout the cytoplasm, in addition to large masses of pigment like those found in the liver.

The cytoplasm of the red corpuscles was stained a brownish colour, suggesting the presence of colloidal iron, while inorganic iron was present in small granules in the nuclei. The nuclei of the leucocytes were stained dark blue.

In tissues taken from *gold-fish* the hepato-pancreas treated with haematoxylin showed two or three deeply-stained small round granules in the cytoplasm of each cell—these granules were independent of the nucleus and were found in unstained sections to be highly refractile and colourless. They were also strongly stained by the ferrocyanide method. Sections of the kidney and ovary showed similar granules, but not to the same degree. In its blood films the red corpuscles were stained brown, presumably owing to the presence of colloidal iron; the rest of the film was unstained.

In the *cray-fish* the hepato-pancreas gave a very well-marked reaction, the granules in the nuclei being more deeply stained than in any other animal tissue examined. There were also well-stained granules scattered throughout the cytoplasm.

In the *gonidia*, the ova showed a well-marked reaction.

The staining was more intense in cray-fish tissues generally than in any of the higher animals studied.

In the *oyster*, the hepato-pancreas and the gills showed numerous granules scattered through the cytoplasm, and well-stained nuclei.

In the *earth-worm*, the granules in the cell nuclei were extremely well shown and the cuticle was also well stained.

In *hydra* the chloroplasts in many of the cells show a well-marked bluish stain.

In *swimming prawns* and "*plankton*," the swimmerettes are stained uniformly deep blue.

In *sagitta*, the body and tail are also stained deep blue.

## Conclusions.

1. Inorganic iron is more widely distributed throughout animal and vegetable tissues than is generally realised.

2. The lower plants and animals give the reaction for inorganic iron much more strongly than do the higher ones.

3. Granules containing inorganic iron are present in almost all the nuclei of plants and animals.

4. Aquatic animals, either marine or fresh water, contain more inorganic iron than those living on land.

5. Foetal tissues contain more inorganic iron than do adult tissues.

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# LIX. THE NOMENCLATURE OF THE SO-CALLED ACCESSORY FOOD FACTORS (VITAMINS).

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In 1912 Hopkins published his classical paper in which he described the important influence of certain dietary constituents on the processes of growth and nutrition. These substances he termed the "accessory factors of the diet." At about the same time Funk, who was working on the subject of experimental beriberi, coined the name "Vitamine" for the same class of substances. Since then the literature has been a good deal confused by the great variety of names which have been utilised to denote these or similar dietary constituents (auximones, Bottomley; nutramines, Abderhalden, etc.). The criticism usually raised against Funk's word Vitamine is that the termination "-ine" is one strictly employed in chemical nomenclature to denote substances of a basic character, whereas there is no evidence which supports his original idea that these indispensable dietary constituents are amines. The word has, however, been widely adopted, and therefore until we know more about the actual nature of the substances themselves, it would be difficult and perhaps unwise to eliminate it altogether. The suggestion is now advanced that the final "-e" be dropped, so that the resulting word Vitamin is acceptable under the standard scheme of nomenclature adopted by the Chemical Society, which permits a neutral substance of undefined composition to bear a name ending in "-in." If this suggestion is adopted, it is recommended that the somewhat cumbrous nomenclature introduced by McCollum (Fat-soluble A, Water-soluble B), be dropped, and that the substances be spoken of as Vitamin A, B, C, etc. This simplified scheme should be quite sufficient until such time as the factors are isolated, and their true nature identified.

# LX. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. III: TECHNIQUE FOR CARRYING OUT FEEDING TESTS FOR VITAMIN A (FAT-SOLUBLE A).

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In a recent communication Osborne and Mendel [1920] referred to the experiments of Steenbock, Boutwell and Kent [1918], and of Drummond [1919, 1] on the destruction of the fat-soluble accessory factor by heat. They were unable to confirm these observations, and in other respects there were also discrepancies for which the reason was not apparent. We have made a careful examination of these discrepancies, and believe that satisfactory explanations are available. Of the difference between the opinions on the effect of heat on the nutritive value of butter we have little to say in this paper, because a later communication will be devoted entirely to that subject; it may, however, be said in passing that we have evidence that the destruction of fat-soluble A is an oxidation process, which confirms what has already been stated by Hopkins [1920]. It is discrepancies of another type which we wish to discuss in this paper, such for instance as the considerable differences noted between the amounts of butter fat required to supply the fat-soluble vitamin for growing rats, to which attention was drawn by Osborne and Mendel [1920]. At first our tendency was to ascribe such differences to variations in the food value of the samples of butter used by the separate investigators, but after giving the matter more careful attention, we have formed the opinion that this undoubted source of disagreement is frequently accompanied by one of a more serious nature, and one, fortunately, that can be readily eliminated. We refer to the very different types of basal diet used in various laboratories. In a previous paper of this series [Drummond, 1919, 2], an experimental method was described by which substances may be tested for the presence of the fat-soluble factor. This method was used for some time with success, but we were led to introduce certain improvements which are described here, and which may be helpful to other investigators who encounter the many difficulties which surround this type of research.

There appears to be little or no need to emphasise the importance of employing carefully selected animals for feeding tests of this nature, for one gathers from the published results that the majority of investigators fully

appreciate this point. In our opinion, however, it is frequently the composition of the basal dietary which is responsible for many of the misleading and contradictory statements which tend to confuse the literature on the vitamins at the present time.

Evidence has been produced which tends to show that the requirements of the growing rat for vitamin A become less as the animal approaches maturity [Drummond, 1919, 2], and our experience leads us to believe that the amount of vitamin which must be supplied to a rat in order to restore growth which has been inhibited by feeding on the deficient basal diet, will be inversely proportional to the weight of the animal.

Should this be confirmed, we think many of the discrepancies in the literature will be accounted for.

## Preparation of Purified Basal Diets.

i. *Preparation of Pure Protein.*

Up to the present we have made a practice of using only highly purified caseinogen as a source of purified protein in our basal dietaries. We have done this following the general scheme used by many other investigators, but we are now considering whether it would not be advisable to employ another protein of equal or superior tissue building value, and one less prone to be contaminated with the fat-soluble factor. A search for such a protein is being made. Commercial caseinogen contains relatively large amounts of the fat-soluble vitamin, and should never be used for experiments relating to that factor without having been carefully purified. We have encountered numerous cases in which prolonged growth of young animals was observed in young rats which were fed upon a diet supposedly free from fat-soluble A, but which were in reality obtaining considerable supplies of the vitamin from the insufficiently purified caseinogen.

We heat our caseinogen for 24 hours or more in shallow dishes to a temperature of 102° C., after which it is subjected to a prolonged and continuous extraction with alcohol and ether.

ii. *Purity of Carbohydrate.*

In the past we have gone to the expense and trouble of preparing a highly extracted form of wheat starch to use as a source of carbohydrate in the basal diets. Later experiments have shown us however that rice starch in the crude form is almost entirely devoid of fat-soluble A, and may be employed without any lengthy and costly preliminary extraction.

iii. *Purity of Fat.*

In the selection of a fat to include in the basal fat-soluble-free diet the greatest care must be employed. As will be shown in the following paper the natural oils and fats which are usually supposed to be free from vitamin A,

are not always so, and disconcerting variations in a single oil may be encountered. As a consequence of many such conflicting results we have carefully reinvestigated this question, and have ascertained that no hard and fast line can be drawn between the animal and vegetable oils and fats, when their growth-promoting powers are being considered. We therefore think it advisable that no natural oil or fat should be used as a source of fat in a diet intended to be free from fat-soluble A. To overcome this difficulty we employ a fully hardened (hydrogenated) and refined vegetable oil, usually cotton seed oil. Such oils consist very largely of tristearin, and are, so far as we can ascertain from carefully controlled feeding tests, entirely devoid of vitamin A. It is possible that even greater security might be obtained by excluding fat entirely from the basal diet, in view of the fact that rats appear to be able to dispense with the presence of pure fats in their diet [Drummond, 1919, 2; 1920].

iv. *Purity of other Constituents of Basal Diet.*

The orange juice and salt mixture which we include in the basal ration have been proved by direct tests to be devoid of vitamin A. The yeast extract is also thought to be equally inactive in that respect, although definite results have not yet been obtained to make quite sure of this point.

The composition of the purified basal ration which we employ in all our routine tests is given below:

| | |
|---|---|
| Purified caseinogen ... ... ... | 18 parts |
| Purified rice starch ... ... ... | 52 ,, |
| Refined hydrogenated vegetable oil | 15 ,, |
| Yeast extract ... ... ... | 5 ,, |
| Orange juice ... ... ... | 5 ,, |
| Salt mixture ... ... ... | 5 ,, |

Rats fed upon this diet behave in somewhat different manner according to their age [Drummond, 1919, 2]. Small rats of 50–70 g. (4–5 weeks old) should show very little growth at all, and should remain stable for a week or two after the slight initial growth. Any considerable increase of body weight in rats of this age when fed upon a purified diet of this composition is interpreted by us as an indication that the basal ration is insufficiently purified. Animals considerably over 100 g. are in our opinion unsatisfactory for testing for vitamin A, and as far as possible we attempt to test all fractions on rats the growth of which has been suspended for 10–14 days, and which are not heavier than 80–120 g.

### Testing Substances for Vitamin A.

As far as possible we make it a routine in this laboratory to test all substances for the presence of vitamins by administering a definite weight of the substance directly to the animal before the day's ration of the basal food is

given. In the large majority of cases this method, which is the only one permitting of an accurate quantitative measure of intake, is practicable. In cases where it is found to be difficult to carry this out, the supplement of foodstuff or fraction must be incorporated in the diet, and the intake judged by records of the total consumption of food.

## Results.

Even with the employment of the greatest care, this method occasionally gives results of doubtful significance. The enormous amount of routine work which is entailed by feeding large numbers of experimental animals by such a process necessitates in an average laboratory some restriction in the size of the experimental groups, a fact which tends to increase the error due to individual variation. Such errors are frequently encountered, and are sometimes disconcerting, but one usually obtains a definite result from the majority of the animals in a particular group.

As we have previously remarked, we are of the opinion that insufficient purification of the basal diet is responsible for many misleading results.

In the curves given by Osborne and Mendel [1920], it will be observed that some of the young rats grew for a considerable time, and attained a fairly heavy weight before they showed the typical decline due to deficiency of vitamin A. This means that when the rats are fit to use for feeding tests they are often considerably over 100 g. in weight. Such animals would presumably recover health and recommence growing on receiving a much smaller amount of the missing vitamin than would be necessary to restore a declining rat of 60–90 g. Further, the former animal, although it had ultimately declined on a deficiency of fat-soluble A, would still be obtaining some fat-soluble factor in the impure basal diet in addition to that contained in the supplement. The observed result might therefore lead one to ascribe a higher food value to the supplement than was justifiable.

Our own experience has given us many examples of how the presence of very small amounts of the factor A in an insufficiently purified basal diet may confuse the issue of the experiment.

## Summary.

In testing foodstuffs for the presence of the fat-soluble vitamin the greatest care should be devoted to ensuring that the basal dietary is rendered as free from that vitamin as possible. Details for the preparation of a highly purified ration are given. Failure to work with a sufficiently pure diet may lead to conflicting and misleading results.

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# LXI. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. IV: NUTS AS A SOURCE OF VITAMIN A.

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The examination of a large number of animal and vegetable fats and oils for the presence of the fat-soluble A accessory food factor suggested to the authors the desirability of investigating raw nuts, known to be rich in fats, for the same vitamin. Certain nut butters had already been examined by Halliburton and Drummond [1917], who had come to the conclusion that they were not equal to butter in this respect, but that there were "certain indications which made it probable that the nuts themselves do in some cases contain the accessory factors in small amount." The literature has not provided accounts of any similar work on the subject, although the vitamin B has for some time been known to occur in large quantities in the coconut and pea-nut [Daniels and Loughlin, 1917; Johns, Finks and Paul, 1919], and, more recently, has been found in the almond, English walnut, Brazil nut and chestnut by Cajori [1920].

## Experimental Method.

The method adopted for the investigation was the one usually followed in this laboratory. Rats of 50–70 g. weight were fed on an unlimited allowance of a basal diet consisting of

| | | |
|---|---|---|
| Purified caseinogen | ... | 18 grs. |
| Purified starch | ... ... | 52 ,, |
| Salt mixture | ... ... | 5 ,, |
| Yeast extract (marmite) | ... | 5 ,, |
| Hardened cotton seed oil | ... | 15 ,, |
| Orange or lemon juice | ... | 5 cc. |

mixed with sufficient cold water to form a stiff paste. Fresh water for drinking and cleaning purposes was supplied daily. The rats were weighed twice a week and when they had ceased growth and their weights had remained practically stationary for four successive weighings, they were fed daily with

approximately 1 g. per rat of the nut to be tested. The eagerness with which the rats came to eat their nuts made this a comparatively simple matter, most of them consuming their ration immediately, and in only one experiment, that of the almonds, were any portions left uneaten on the following morning.

## Results.

The weights in g. of the rats on the nuts tested are given in the following table.

| Nuts | | Number of days after commencement of nut supplements | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 4 | 7 | 10 | 14 | 18 | 21 | 25 | 28 | 32 |
| Pea-nuts ... 38·6 % fat | Rat 80 ♀ | 110 | 120 | 118 | 117 | 114 | 109 | 109 | 110* | 110 | 110 |
| | „ 81 ♀ | 80 | 86 | 81 | 86 | 92 | 91 | 91 | 100 | 101 | 100 |
| | „ 82 ♀ | 78 | 82 | 79 | 79 | 81 | 78 | 80 | 77* | 80 | 74 |
| Brazil nuts ... 66·8 % fat | „ 83 ♂ | 107 | 120 | 120 | 99 | 102 | 118 | 120 | 120 | | |
| | „ 85 ♀ | 92 | 100 | 97 | 99 | 99 | 100 | 99 | 102* | | |
| | „ 84 ♀ | 80 | 90 | 89 | 90 | 95 | 94 | 95 | 98* | | |
| Butter nuts ... 60 % fat ... | „ 55 ♀ | 72 | 77 | 71 | 67 | 70 | | | | | |
| | „ 54 ♀ | 88 | 92 | 93 | 92 | 90 | | | | | |
| | „ 56 ♀ | 86 | 104 | 104 | 107 | 104 | | | | | |
| Barcelona nuts | „ 80 ♀ | 110 | 120 | 118 | 125 | 123 | | | | | |
| | „ 81 ♀ | 100 | 100 | 107 | 109 | 109 | | | | | |
| | „ 82 ♀ | 74 | 62 | 70* | | | | | | | |
| Almonds 55 % fat | „ 83 ♂ | 122 | 124 | 130 | 140 | 130* | | | | | |
| | „ 85 ♀ | 106 | 101 | 100 | 101 | 98 | | | | | |
| | „ 84 ♀ | 93 | 94 | 96 | 91 | 89 | | | | | |
| Walnuts ... 62 % fat | „ 55 ♀ | 76 | 79 | 78 | 81 | 81 | | | | | |
| | „ 54 ♀ | 100 | 102 | 105 | 101 | 102 | | | | | |
| | „ 56 ♀ | 113 | 115 | 122 | 115 | 112 | | | | | |

Rats 80, 82, 84, 85, 83 developed a bad form of the characteristic eye disease associated with a deficient supply of vitamin A at points marked *.

## Discussion.

The results are interesting in view of the opinion which is steadily gaining ground that vitamin A is found most abundantly in actively assimilating green plant tissues and to a much smaller extent in resting tissues, such as occur in all seeds, and the special food storehouses in the roots and stems of certain plants. The nuts used were chosen somewhat at random from those usually considered desirable by vegetarians, many of them, as shown in the table, having a high fat content. Structurally, they show some variation. The almond, walnut, pea-nut and Barcelona nut, although not strictly speaking all true "nuts," are true seeds and show well-defined plumule and radicle, while the mass of the seed is filled with cotyledons containing the reserve food store. The Brazil nut has a mass of endosperm in which are embedded the growing points, not fully enough developed to be visible to the naked eye; and the butter nut has probably a similar structure. As the results

showed an almost complete absence of vitamin A from all the six kinds of nuts, no distinction can be drawn between tissues which are purely reserve tissues and those which have potentially another function, such as assimilation, as have the cotyledons of the almond. This suggests that the vitamin must be developed at some stage subsequent to germination, and the authors hope shortly to publish results of work which support this view.

In view of the recent work on the thermostability of vitamin A, another point had to be investigated. Inquiry in one direction showed that pea-nuts are "roasted" before being sent to the market, though at what temperature or for what length of time, no exact information could be obtained. On the other hand another source of information denied any roasting of the nuts previous to their appearance on the market. English walnuts are dried in the open air, but there are no grounds for thinking that this process or that which Brazil nuts, Barcelona nuts, butter nuts and almonds normally undergo before they are sold as food causes any destruction of vitamin A.

The nuts were tested for the presence of the lipochromes, carrotene and xanthophyll, by the method described in the previous paper on fats and oils by the authors. In no case was more than a mere trace of pigment found, and this failed to give characteristic lipochrome reactions with strong sulphuric acid, nitric acid, and iodine in potassium iodide solution.

## Conclusions.

1. The nuts examined, Brazil, Barcelona, pea, walnuts, almonds and butter nuts, although containing large percentages of fats, possess a relatively low food value as sources of the vitamin A often associated with fats.

2. These results furnish additional evidence for the theory that vitamin A is formed in the green part of the living plant and is not stored to any appreciable extent as such in the seed and other resting tissues.

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# LXII. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. V: THE NUTRITIVE VALUE OF ANIMAL AND VEGETABLE OILS AND FATS CONSIDERED IN RELATION TO THEIR COLOUR.

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More than one investigator has recently called attention to the apparent relationship between the presence of certain yellow pigments in foodstuffs and the occurrence of the fat-soluble accessory factor.

Steenbock [1919] traced this association in a number of natural foodstuffs, but also pointed out that certain exceptions occurred in which the presence of the fat-soluble factor was not associated with yellow pigments. To cover such exceptions he suggested that the colouring matters might be present in the form of a leuco-compound. This theory was criticised by Palmer and Kempster in an interesting series of papers [1919, 1, 2, 3], in which they showed that the natural yellow pigment of fowls, which is derived from the xanthophyll of the foods, bears no important relation to growth, or to the functions of fecundity or reproduction, at least for one generation.

Rosenheim and Drummond [1920] showed that the experiments of Palmer and Kempster were open to some criticism, and that certain of their results might be interpreted as evidence that the lipochrome pigments played a rôle in nutrition. In this paper some support was given to the theory advanced by Steenbock, but it was definitely shown that fat-soluble A is not identical with either of the two chief lipochromes, carrotene and xanthophyll.

In one criticism advanced by Palmer [1919] against the association theory he pointed out that certain vegetable oils (*e.g.* cottonseed oil), which are supposed to be rich in lipochromes, are destitute of the vitamin.

This criticism appeared to be a reasonable one and stimulated us to re-examine the nutritive values of certain animal and vegetable oils and fats. It was obvious that a useful index of the food value of such fats would be in our possession, if it were definitely found that the presence of the fat-soluble factor is associated with certain yellow pigments. In order to test the accuracy

of Palmer's criticism we decided to investigate the growth-promoting power of a vegetable oil which is deeply coloured with lipochrome pigments, namely, palm oil.

Several young rats which were in failing health, as a result of continued maintenance on a diet deficient in vitamin A, were given a diet containing 20 % of a deeply pigmented sample of palm oil. The change of diet brought about an immediate and striking recovery; in fact, one rat of this series showed a more rapid recovery of health and clearing up of the well-known eye disease than we had ever before encountered.

As soon as this result was confirmed, it was obvious that the whole question of the relative food value of the animal and vegetable oils must be reinvestigated. The earlier experiments, made before the technique of conducting such feeding tests was as good as it is now, had led us to consider that the vegetable oils as a class contain little or none of the factor A, whereas animal fats, with the exception of lard, are good sources of this indispensable substance [Experiments and bibliography, Halliburton and Drummond, 1917].

Accordingly a large number of animal and vegetable oils, several of which had not been previously investigated, were tested, both for the presence of the growth-promoting factor A, and for the presence and nature of any accompanying pigments. The results, which are given in tabular form in Table I (and in Curves 1–22, Figs. 1, 2 and 3), demonstrate clearly that we have not been justified in drawing a hard and fast line between animal and vegetable oils and fats, when considered from the point of view of their vitamin content.

Many of the vegetable oils contain appreciable amounts of the A factor, although as a class they are considerably inferior to the majority of animal fats, such as butter or cod liver oil. Our experiments make it clear, however, that unless we accept the suggestion advanced by Steenbock, of the existence of a leuco-compound of the pigment, to account for the exceptions, the theory of the association of yellow pigments of the lipochrome class with the presence of vitamin A fails to hold good. This is particularly well seen in the case of one or two animal fats, such as the pigment-free dog fat, which we found to be a relatively rich source of A (Curves 11 and 12, Fig. 1).

### The Presence of the Fat-soluble Accessory Factor in certain Vegetable Oils.

Our early results with palm oil naturally caused us to devote considerable attention to the vegetable oils. It has been suggested by McCollum, Simmonds and Pitz [1916] that the fat-soluble factor may exist in certain plant tissues in the form of a complex, which must be decomposed before the fat-soluble factor can be obtained in a truly "fat-soluble" form, a process which appears to be brought about very readily, as by treating the crushed seed with alcohol. The amount of the fat-soluble factor in oils derived from seed will therefore depend upon the amount originally present in the seeds, and

the treatment which the seeds have undergone prior to the extraction of the oil. Thus, in considering our results with palm oil, it must be remembered that the majority of the samples at present obtainable have been prepared by the crude native process. This consists in throwing the broken fruit into pits dug in the ground and lined with leaves, where they are left for some days until the flesh softens, so that the kernels may be readily separated. During this time the flesh undergoes fermentation processes, and the oil is finally

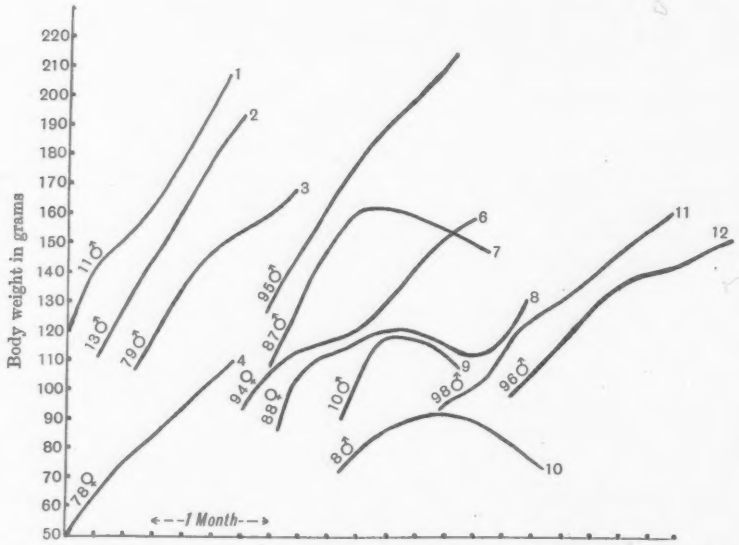


Fig. 1. In every case the curves show only the behaviour of the animal after the supplement has been given. The preliminary period during which growth was inhibited on a deficiency of vitamin A is omitted for simplicity.

Curves 1 and 2. Rapid recovery of growth by addition of stable-fed horse fat equivalent to 10 % of the diet.

Curves 3 and 4. Slightly less rapid recovery on receiving the same amount of a deeply pigmented sample of grass-fed horse fat.

Curves 5 and 6. Recovery of growth on supplement of 15 % abdominal fat of pig (there was one marked failure to recover in this series).

Curves 7 and 8. Evidence that mutton fat contains small amounts of vitamin A. Results of feeding a supplement of 15 %.

Curves 9 and 10. Failure to recover on 15 % subcutaneous fat of pig.

Curves 11 and 12. Fairly good recovery maintained by a supplement of 15 % of dog-body fat; a pigment-free fat.

separated by beating the residue to a pulp and straining. Whether the vitamin exists free in the flesh of the palm nut, or whether it is present as a complex, from which it is liberated during the fermentation, is not known, but experiments to determine this point are contemplated.

The pigment of palm oil is very largely carrotene, accompanied by a small

amount of xanthophyll. Of the other vegetable oils examined, only maize and linseed gave definite tests indicating the presence of appreciable quantities of carrotene and xanthophyll.

Table I.

| Oil or fat | Remarks | Rough approximation of value as source of vitamin A | Lipochrome pigments | | |
|---|---|---|---|---|---|
| | | | Total | Carrotene | Xanthophyll |
| Butter ... ... | Average sample ... ... ... | 10 | 10 | 8 | 2 |
| „ ... ... | Dark sample (Darlington) ... | 10 | 30 | 24 | 6 |
| „ ... ... | Pale „ „ ... ... | 10 | 15 | 12 | 3 |
| „ ... ... | "Strawberry," 1 week winter feed | 10 | 10 | 8 | 2 |
| „ ... ... | „ 2 weeks „ „ | 8 | 2·5 | 2 | 0·5 |
| „ ... ... | „ 2 weeks grass ... | 10 | 11 | 8 | 3 |
| Cod liver oil ... | Average sample ... ... ... | 10 | — | — | — |
| Dog-body fat ... | Subcutaneous ... ... ... | 6–7 | 0 | | |
| Beef fat ... ... | „ ... ... ... | 6–8 | — | | |
| Mutton fat... ... | Average ... ... ... ... | 2 | 0 | | |
| Pig fat ... ... | Subcutaneous ... ... ... | 1 | 0 | | |
| „ ... ... | Perinephritic ... ... ... | 5–6? | 0 | | |
| Lard ... ... | Refined ... ... ... ... | 0 | 0 | | |
| Horse fat ... ... | Stable fed ... ... ... ... | 6–8 | 1 | 1 | 0 |
| „ ... ... | Grass fed ... ... ... ... | 6–7 | 65 | 50 | 15 |
| Linseed oil... ... | Average sample ... ... ... | 1–2 | 16 | 6 | 10 |
| Hardened linseed oil | ... ... ... ... | 0 | 0 | | |
| Palm oil ... ... | Very dark ... ... ... ... | 3–4 | 76 | 57 | 19 |
| Maize oil ... ... | Bright yellow ... ... ... | 2–3 | 46 | 23 | 23 |
| Cottonseed oil ... | Average sample ... ... ... | 1 | 0 | | |
| Hardened cottonseed oil ... ... | „ „ ... ... ... | 0 | 0 | | |
| Peanut oil ... ... | „ „ ... ... ... | 1 | 0 | | |
| Sesame oil ... ... | „ „ ... ... ... | 0 | 0 | | |
| Olive oil ... ... | „ „ ... ... ... | 0–1 | 0 | | |

*Note on Table I.* The figures which are given to represent the approximate food values must be regarded as very empirical. It is obvious that our technique is as yet insufficiently accurate to enable us to allocate relative food values. These numbers, based on butter as 10, are given, however, to indicate very roughly the order of the differences in the nutritive values of the various oils and fats, so that it can be seen that they do not run proportional to the amount of lipochrome pigments, and only refer to the actual samples examined.

The figures representing the amount of pigments present in the oils are derived from colorimetric estimations made on the unsaponifiable fraction from 10 g. of the oil. This fraction dissolved in 25 cc. of light petroleum was compared with a standard solution of potassium dichromate in a Hellige colorimeter. This method is one given by Wilstätter, and gives good results for estimations of carrotene and xanthophyll, when standard curves have been prepared from pure solutions of those substances. The proportions of carrotene and xanthophyll present were judged approximately by colour comparisons of the respective fractions obtained by the well-known phase-test with 80 % alcohol and light petroleum.

The pigments of cottonseed oil, sesame oil and peanut oil appear to be of a different class.

### The Fat-soluble Factor in Animal Fats.

Following our observation of the presence of the vitamin in highly-pigmented palm oil, we decided to test the food value of certain pigment-free animal fats. As will be seen from Curves 11 and 12, a sample of dog-body fat, which was devoid of pigment, showed considerable growth-promoting activity. This led us to re-examine the food value of lard. We again confirmed the absence of the fat-soluble factor from a highly-refined sample of lard, which was also pigment-free. When, however, we examined the fresh pig-body fat, purchased from a neighbouring butcher's shop, we obtained rather conflicting results, which indicated that this fat may contain the fat-soluble factor, particularly in the case of a sample from the abdomen (Curves 5, 6, 9, 10). Both samples were devoid of pigment.

As we knew nothing of the origin of these samples we felt that the results were insufficiently conclusive to enable us to arrive at any decision on what is obviously a most important point. We are, however, carrying out a number of feeding experiments on pigs, in conjunction with Capt. J. Golding, of University College, Reading, and Dr S. S. Zilva, of the Lister Institute, the results of which will shortly be published, and which we hope will provide a solution to the much debated question of the food value of lard.

### Food Value and Pigmentation of Animal Fats.

The valuable researches of Palmer and Eccles [1914] have taught us much concerning the origin of the lipochrome pigments of the body fats and milk fats of animals. When we were examining the possibility of vitamin A being associated with the lipochrome pigments, we paid careful attention to their work. It will be remembered that by feeding a cow on a diet deficient in lipochrome pigments, they were able to obtain a butter almost devoid of pigment. As a result of the very kind co-operation of Dr L. Hamilton, Warden of Studley Agricultural College, Warwickshire, we were enabled to make a similar observation. A shorthorn cow which had been out at grass since the early part of the year was placed in stall on May 12th, and was given a winter feed, consisting of 80 lb. of mangolds, 50 lb. hay, 8 lb. cottonseed cake and 5 gallons of water daily. After seven days indoors the milk was collected for four days, and the cream made into butter. It was of approximately the same depth of colour as that made from the milk of the cows still out at pasture. After 14 days indoors the milk was again collected for a few days and made into butter. This sample was much paler than the previous one and of a much more granular consistency. The cow was then turned out of doors, and after two weeks more her milk again collected separately and made into butter. This had regained its normal colour and consistency. Quantitative measurements of the pigments present in each sample of butter (Table I) and of its growth-promoting activity were made. The latter were carried out by the technique described in the previous paper, substituting 3 % of the butter for

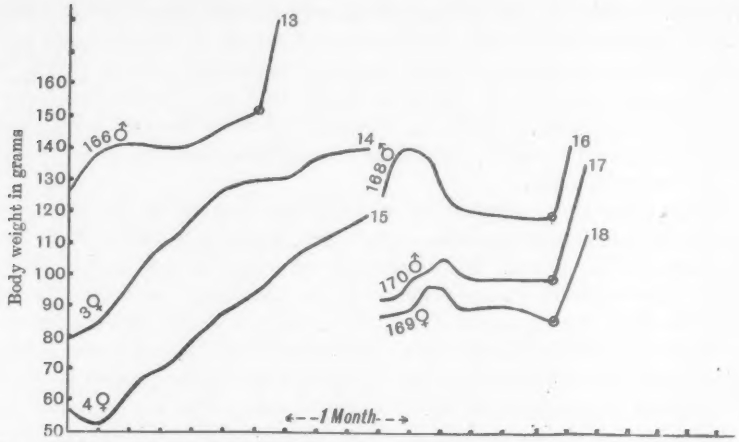


Fig. 2. Preliminary period on basal diet is not represented, all curves begin at point at which supplement was given.

Curves 14 and 15. Show prompt recovery with a supplement of 20 % of a deeply-coloured sample of palm oil. One rat of this series, before it was given palm oil, was declining in weight and showed very severe external eye disease. This improved rapidly when the palm oil was given.

Curves 13 and 16. A supplement of 15 % cottonseed oil has no marked beneficial effect on growth. From point ⊙ 10 % butter given instead of cottonseed oil.

Curves 17 and 18. Failure of supplement of 15 % of sesame oil to promote growth. From point marked ⊙ 10 % butter given.

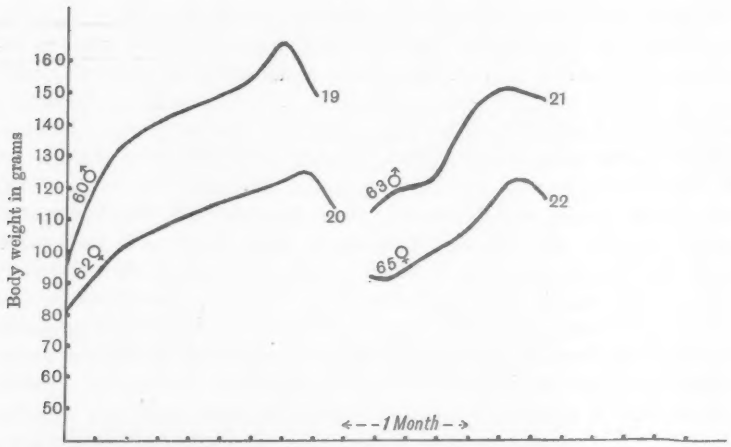


Fig. 3. The preliminary period on fat-soluble-free basal ration is omitted.

Curves 19 and 20. Some recovery, although temporary, on adding a supplement of 20 % of maize oil (highly pigmented).

Curves 21 and 22. Same type of recovery on 15 % of linseed oil.

the larger quantity of the hardened fat in the basal diet, this quantity being selected because experiments with an average sample of mixed butter had shown that it was just appreciably above the minimum at which growth is obtained, and therefore any falling off in the food value would be quickly observed. The control, in which 3 % of one of the butters was used in addition to 12 % of the hardened fat, showed no better growth than was given by the 3 % butter series alone.

It was found that corresponding with the large decrease in the amount of pigment in the butter there was a slight but appreciable falling off in the growth-promoting power. When put back on grass the cow gave a richly coloured butter, and there was at the same time a rise in its food value (Fig. 4). These results at first appeared to lend some support to the pigment theory, for they were obtained before the experiments with the colourless active animal fats were completed, but we observed a lack of proportion between the decrease of pigmentation and that of food value. This was confirmed by our experience with two samples of butter fat sent to us by Capt. J. Golding from the Royal Agricultural Show held in June this year at Darlington. By his kindness we obtained samples of the darkest and palest butters from the butter-making competitions held there. Neither sample contained added pigment. Our feeding tests showed that the growth-promoting power of the lighter coloured sample, which had a pale cream tint, appeared to be as good as, if not slightly better than, that of the darker sample, which possessed an unusually deep orange yellow colour (Table I). We were not able to obtain information as to the feeds of the cows from which the respective butters were made.

Similar results were given by two samples of horse fat, which were obtained from the carcasses of stable-fed and grass-fed horses respectively (Curves 1, 2, 3 and 4, Fig. 1). The results obtained by Palmer and Eccles [1914, 1, 2, 3, 4], and by Palmer [1915, 1916], are of value in an attempt to interpret these results. The latter paper showed that a definite physiological relation exists in all species of animals between the pigmentation of tissue fat with lipochrome pigments and the presence of these pigments in the blood serum. Those species, such as the cow, horse, hen, and man, the tissue fat of which is coloured with lipochrome, transport these pigments in their blood serum. Species, the tissue fat of which is colourless, such as the pig, sheep, and goat, carry only insignificant traces of lipochrome pigments in the serum. Certain unpublished experiments, made in conjunction with Dr O. Rosenheim, have shown us, however, that in both classes of animals considerable amounts of lipochromes are normally to be found in the liver. It therefore appears probable that in the digestion and absorption of plant foods such as green vegetables, which contain not only lipochromes, but also fat-soluble accessory factor, both vitamin and pigment pass to the liver, and may be held there in considerable amounts. What significance these observations may have is as yet uncertain.

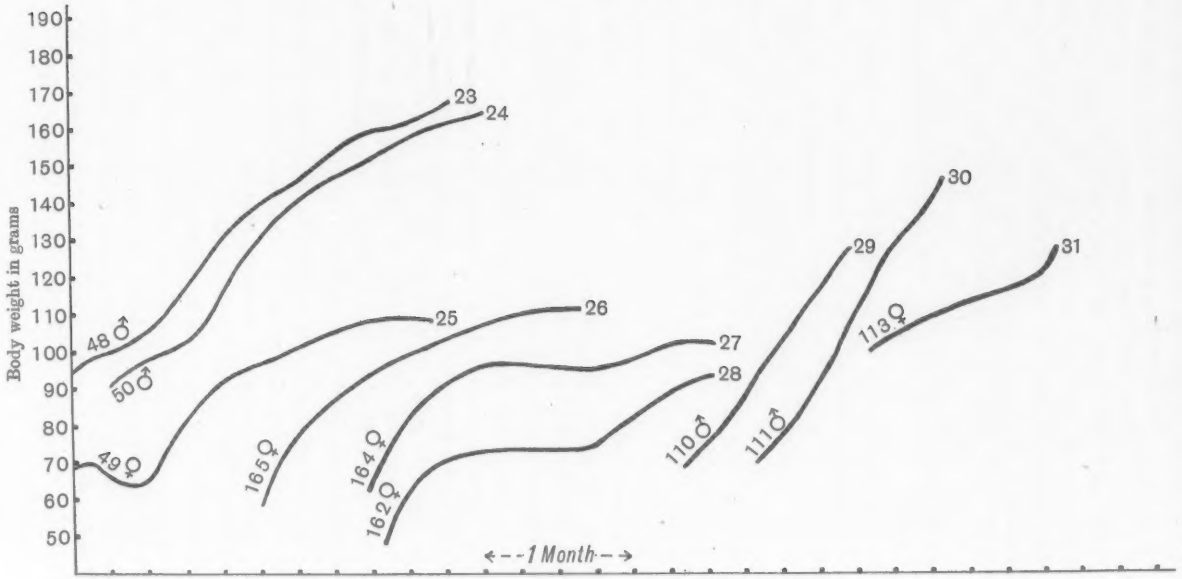


Fig. 4. The preliminary period of inhibited growth on the basal ration is omitted.

Curves 23–25. Recovery on being given supplement of 3 % of butter from shorthorn cow, "Strawberry," after one week's winter feeding.

Curves 26–28. Recovery somewhat less complete on butter from "Strawberry," after 14 days' winter feeding.

Curves 29–31. Recovery more rapid than in either above cases on supplement of 3 % of butter from "Strawberry," after 14 days' grass feeding.

Whether there will be any transference of the pigment to the body fat and subsequent storage there, will depend, as Palmer has shown, on the species of the animal, but probably all mammals are able to store up the fat-soluble factor to a greater or less extent in the fat depôts of their body. In other words, the fat-soluble vitamin and the lipochromes do not appear to follow the same paths after absorption into the animal organism. The experiments on pigs now in progress, to which reference has been made, will, it is hoped, provide more definite information on this point. No success has followed attempts to cause storage of lipochromes in the body fat of swine by feeding them on a diet rich in green fodder, and it remains to be seen whether such fats contain vitamin A.

The experiments outlined in this communication make it apparent that considerable caution must be observed in allocating a food value to an oil or fat, unless the sample has been tested by direct quantitative feeding experiments. On the whole the edible animal fats, such as butter and cod liver oil, appear to be of greater value in assisting growth than the common vegetable oils. But the idea that the latter are as a class nearly or completely devoid of fat-soluble A must be modified. What appears to us to be of primary importance is the appreciation of the fact that the nutritive value of a fat of animal origin is dependent on the diet of the animal from which it is derived, and that the food value of either animal or vegetable oils and fats may be considerably affected by the methods of preparation and refining. In agreement with the observations of Rosenheim and Drummond [1920], who demonstrated that fat-soluble A is not identical with either carrotene or xanthophyll, we have now found that the presence of these lipochrome pigments in animal or vegetable oils and fats is neither a measure, nor even a reliable indication, of growth-promoting powers, as may be seen by comparing the estimations of pigments given in Table I with the growth curves. It remains to be seen whether there are any grounds for believing that the fat-soluble accessory factor is a pigment which may in some cases exist in a leuco-form, as suggested tentatively by Steenbock.

The research also gives us a preliminary result on the important question of the influence of winter feeding of cows on the food value of the butter they yield. In the short space of three weeks it was found that a diet low in vitamin A brought about a distinct fall in the nutritive value of the milk secreted. Experiments on a larger scale are already in progress, from which it is hoped to obtain more accurate and definite information on this important point.

We are indebted to Dr Rosenheim of King's College, London, for the loan of a Hellige colorimeter, and for standard curves which he had prepared. The expenses of this research were defrayed by a grant from the Medical Research Council, which the authors wish gratefully to acknowledge.

SUMMARY.

1. No hard and fast line can be drawn between the animal and the vegetable oils and fats when their value as a source of vitamin A is being considered.

2. Taken as a class the animal fats possess a growth-promoting power superior to that of the vegetable oils, but we have observed that one or two members of the latter class (*e.g.* palm oil) may show considerable activity in that respect.

3. Unless we assume the existence of a leuco-form, it does not appear probable that the fat-soluble vitamin is a member of the lipochrome class of pigments. The frequent association of the growth factor with pigments of that type must therefore be regarded as accidental.

4. The nutritive value of an animal oil or fat would appear to be influenced considerably by the diet of the animal. One preliminary experiment shows that the winter feeding of cows may have the effect of lowering the food value of the milk unless considerable care is exercised in the selection of the animal's diet.

Probably the nutritive value of both animal and vegetable oils and fats is influenced by the processes of preparation and refining which they may undergo.

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